Aus dem Institut für Virologie
Direktor: Prof. Dr. Stephan Becker
Des Fachbereichs Medizin der Philipps Universität Marburg

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**Inaugural-Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften
(Dr. rer. nat.)**

dem Fachbereich Medizin der Philipps Universität Marburg vorgelegt von

Jan Baumann
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Angenommen vom Fachbereich Medizin der Philipps Universität Marburg am:
28.06.2016

Gedruckt mit Genehmigung des Fachberreichs.

Dekan: Prof. Dr. Helmut Schäfer
Referent: Dr. Mikhail Matrosovich
1. Korreferent: Prof. Dr. Pleschka

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Summary

The transmission of influenza A viruses from avian to other species involves numerous adaptive processes to overcome the species barrier. One major determinant of host-range restriction is the viral hemagglutinin (HA). HA plays a crucial role in virus entry into the host cell by mediating receptor-binding and membrane fusion. Virus adaptation to mammals results in alteration of receptor-binding specificity. There is growing evidence that the HA-mediated membrane fusion activity contributes to host range restriction as well. This study aimed to identify host specific differences in membrane fusion properties and to characterise potential alterations during interspecies transmission.

In the first part of the thesis Eurasian avian-like swine viruses that emerged by transmission of an avian H1N1 virus in pigs in the late 1970s in Europe were shown to have a higher pH optimum of HA-mediated fusion (pH 5.1-5.4) and a decreased HA stability when compared to avian precursors (pH 4.9-5.2). These results indicate that this avian-to-swine transmission was accompanied by changes in HA stability. Sequence comparison revealed eight amino acid substitutions that separate the HA of early avian-like swine viruses from their putative avian precursor. Furthermore, mutations in one of these positions contribute to the low stability phenotype. In agreement with natural avian-to-swine transmission, experimental adaptation of a potential avian precursor of the avian-like swine lineage to pigs resulted in a decreased HA stability. This states the first formal proof that viral membrane fusion and stability properties change during interspecies transmission.

The second part of the thesis investigated differences in membrane fusion activity among different avian virus species. Comparison of H7 viruses from wild birds and domestic poultry suggests that Eurasian H7 poultry viruses have a higher pH optimum of membrane fusion (pH 6.2) and thus possess a lower stability than H7 viruses from wild birds (pH 5.2). Moreover, all tested Eurasian H7 viruses express a lower HA stability than HAs from other subtypes (H2, H3, H4, H5, H13, H14 und H16). Previous studies indicate that H5 viruses with low HA stability replicate but do not transmit via respiratory droplets in the ferret model (Imai et al., 2012; Herfst et al., 2012). Thus, it

is feasible, that H7 viruses originated from poultry are restricted in ferrets and humans to similar extend.

In the last part, fusion properties of human pandemic and zoonotic viruses were studied. HAs of pandemic viruses from the last century initiated fusion in a narrow pH range between pH 5.0 and 5.2. In contrast, the swine-origin 2009 pandemic virus HA starts to fuse at a pH 0.2 units higher, which might be due to the swine origin of this HA. This further suggests that fusion characteristics continue to adapt in the course of subsequent circulation. The pH optimum of fusion of a zoonotic human H7N9 (2013) virus represents an intermediate in that it is lower when compared to putative ancestors circulating in wild birds, but still higher than that of typical human-adapted viruses. This may account for limited human-to-human transmission observed for this virus.

In order to further investigate which changes in HA are needed for the emergence of avian viruses in humans, HA substitutions separating the 1968 Hong Kong pandemic virus HA from the putative avian precursor were examined. In addition to the well-known switch in receptor specificity, binding avidity changed prior to or during the emergence in humans. In this study, no difference in viral stability was observed between the pandemic virus and the putative avian precursor. This indicates that the avian ancestor was already sufficiently stable to facilitate replication and transmission in humans.

In sum, this study shows that membrane fusion properties vary between host species and alter during influenza A virus emergence in new hosts. This suggests HA-mediated fusion and HA stability to act as host range restriction factors. Alterations in membrane fusion activity and viral stability may not be essential for initial infection of new host individuals. However, an optimal stability seems to be necessary to facilitate transmission within populations of new host species.

Zusammenfassung

Die Anpassung aviärer Influenza A Viren an neue Wirtsspezies umfasst eine Vielzahl von adaptiven Prozessen. Das Glykoprotein Hämagglutinin (HA) des Influenza A Viruses stellt dabei eine der Hauptdeterminanten der Wirtsrestriktion dar. Dies lässt sich auf die essentielle Rolle des HAs während des viralen Zelleintrittes zurückführen, wobei es sowohl die Bindung an den zellulären Rezeptor als auch die Fusion der viralen mit der endosomalen Membran vermittelt. Die Adaptation an die meisten Säugerspezies, inklusive Schwein, Frettchen und Mensch, resultiert in einer veränderten Rezeptorspezifität. Jüngste Studien deuten darauf hin, dass die HA-vermittelte Membranfusion ebenfalls zur Limitierung des Wirtsspektrums beiträgt.

Das Ziel der vorliegenden Arbeit war es wirtsspezifische Unterschiede in der Membranfusionsaktivität von Influenza A Viren zu identifizieren. Dabei sollten potentielle Veränderungen der Fusionseigenschaften während einer Übertragung auf neue Wirtsspezies charakterisiert werden. Hierbei lag der Fokus auf der Transmission sowohl zwischen Vogel und Schwein, als auch von tierischen Influenza A Viren auf den Menschen.

Im ersten Teil der Arbeit wurde die Fusionsaktivität eurasischer aviär-ähnlicher Schweineviren untersucht, welche im Europa der späten 1970er Jahre bei der Transmission eines aviären H1N1 auf Schweine entstanden. Dabei konnte gezeigt werden, das aviär-ähnliche Schweineviren ein höheres pH Optimum der HA-vermittelten Fusion und eine geringere HA-Stabilität aufweisen als ihre aviären Vorläufer. Dies deutet darauf hin, dass die Übertragung vom Vogel auf das Schwein mit einer Veränderung der HA-Stabilität einhergeht. Mittels Vergleich der HA Aminosäuresequenz früher aviär-ähnlicher Schweineviren mit ihren potentiellen aviären Vorläufern konnten acht Aminosäureunterschiede identifiziert werden. Es konnte gezeigt werden, dass eine dieser Mutationen zu der verringerten HA-Stabilität beiträgt. Übereinstimmend mit diesen Ergebnissen resultierte die experimentelle Adaption eines aviären Viruses an das Schwein in einer verringerten HA-Stabilität. Dies ist der erste experimentelle Nachweis, dass die virale Membranfusions- und HA-Stabilitätseigenschaften sich im Zuge einer Interspeziesübertragung verändern.

Der zweite Teil der Arbeit behandelt Unterschiede in der Membranfusionsaktivität von Viren verschiedener aviärer Spezies. Hierfür wurden H7 Viren verglichen die aus wild

lebenden Wasservögeln und domestiziertem Geflügel isoliert wurden. Eurasische H7 Geflügelviren wiesen dabei ein höheres pH-Optimum der Fusion und damit eine geringe HA-Stabilität auf als H7 Viren aus wild lebenden Wasservögeln. Verglichen zu repräsentativen Viren anderer Subtypen (H2, H3, H4, H5, H13, H14 und H16) konnte für alle getesteten eurasischen H7 Viren eine geringere Stabilität beobachtet werden. Erste Studien mit H5 Viren zeigen, dass eine geringe HA-Stabilität eine Aerosoltransmission im Frettchen-Model limitiert. Da humane Influenza Viren vorwiegend über Aerosole übertragen werden, wäre es möglich, dass die geringe Stabilität von H7 Geflügelviren eine Übertragung auf den Menschen beschränkt.

Im letzten Teil wurden die Fusionseigenschaften pandemischer und humaner zoonotischer Viren untersucht. Hämagglutinine pandemischer Viren des letzten Jahrhunderts induzieren Fusion in einem engen pH-Bereich zwischen pH 5.0 und 5.2. Im Gegensatz dazu fusioniert das HA der Schweinegrippe von 2009 bei einem um 0.2 pH Einheiten höheren pH. Dies könnte auf den porzinen Ursprung dieses HAs zurückzuführen sein, was darauf hindeutet, dass die viralen fusogenen Eigenschaften einer fortgesetzten Adaption während einer andauernden Zirkulation in Schweinen unterliegen. Das pH-Optimum der Fusion eines zoonotischen H7N9 (2013) Virus weißt einen intermediären Phänotyp auf. Es zeigt einen geringeren pH der Fusion als putative Vorläufer in wild lebenden Wasservögeln, fusioniert jedoch bei einem höheren pH-Wert als typische human-adaptierte Viren. Dies könnte zu der beobachteten geringen Mensch-zu-Mensch Übertragung dieses Virus beigetragen haben.

Um zu untersuchen welche Adaptionen im HA essentiell für die Anpassung aviärer Viren an den Menschen sind, wurden die Aminosäuresubstitutionen, welche das HA des pandemischen 1968 Virus von seinen möglichen aviären Vorläufern unterscheidet, näher analysiert. Zusätzlich zu dem bereits beschriebenen Wechsel der Rezeptorspezifität konnte eine Veränderung der Rezeptorbindungsstärke beobachtet werden, welche während oder vor Übertragung auf den Menschen erworben wurde. Während dieser Untersuchung konnten keine Unterschiede in der viralen Stabilität zwischen dem pandemischen und dem putativen aviären Vorläufer detektiert werden. Dies deutet darauf hin, dass der aviäre Vorläufer bereits eine ausreichende Stabilität besaß um Replikation und Transmission im Menschen zu gewährleisten.

Zusammenfassend zeigt diese Studie, dass sich die viralen Membranfusionseigenschaften von Influenza A Viren bei der Übertragung auf eine neue Wirtsspezies anpassen. Das deutet darauf hin, dass die HA-vermittelte Fusion und

die HA-Stabilität als Wirtsrestriktionsfaktoren fungieren. Veränderung der viralen fusogenen Eigenschaften könnten essentiell für eine initiale Infektion neuer Wirtsspezies sein. Damit scheint eine optimale virale Stabilität benötigt zu werden um Transmission zwischen Individuen einer neuen Wirtspopulation zu gewährleisten.

1 Introduction

1.1 Historical overview of influenza

Influenza viruses have posed a threat to humans for ages (reviewed in Lina, 2008; Morens and Taubenberger, 2010; Morens et al., 2010). Unspecific clinical signs complicated the identification of the causing disease. Even though records of past pandemics describe diseases with clinical manifestation typical for influenza infection, the source of the diseases remains obscure.

One of the first recorded cases of contagious infection of the upper respiratory tract including influenza-like symptoms can be found in Hippocrates' 'Sixth Book of the Epidemics'. However, the hypothesis that an influenza virus may be the causing agent remains highly speculative. Several European reports suggest a pandemic affecting France, Italy and England between 1173 and 1174. However, the universal use of 'plaque' for epidemics with significant mortality in this time hampers the retrospective reasoning.

The term 'influenza' or 'influenza di freddo' (cold influence) was first recorded in 1357, when an epidemic wave hit Florence in Italy. Later, in 1414, a large epidemic in France was described to rise like a "smelly and cold wind" affecting everyone from the poorest to the rich. The earliest documented outbreak generally accepted as a real influenza pandemic emerged in February 1427 in Southern Europe.

Between 1500 and 1900, records about epidemic diseases became more detailed. The first well-described outbreak was documented in 1580. In the summer of that year, a contagious disease spread from Asia via Africa to Europe, resulting in a significant number of deaths in Spanish, French and Italian cities. Since then, the term 'influenza' has been used to describe such massive epidemics. In the 18th century, a global pandemic originating in China spread to Russia and subsequently westwards across Europe, reaching North America in the spring of 1781. This event was followed by further epidemics in the course of the 19th century (1790, 1803, 1817, 1830 and 1837). In 1889 an epidemic emerged in Russia with a high prevalence, infecting 40% of the population. Isolation of a so far unknown bacterium from patient sputum resulted in the discovery of *Haemophilus influenza* by Pfeiffer. For many years this bacterium was believed to represent the causative agent of influenza.

The first influenza A virus was cultivated in vitro more than ten years after the 'Spanish flu' of 1918. In 1931 the first influenza virus was isolated from pigs, followed by amplification of the first human influenza virus in 1934 (A/Puerto Rico/8/1934(H1N1)). Both viruses are descendants of the 1918 pandemic virus.
Further global pandemics occurred within the last 100 years (1957, 1968 and 2009) and caused millions of deaths. The identification of the influenza A virus led to intense and continuing research (reviewed in Cox et al., 2004; Lina, 2008).

1.2 Influenza A virus taxonomy

Influenza A viruses form one out of five genera of the family *Orthomyxoviridae*. These five genera, influenza virus A, influenza virus B, influenza virus C, Thogotovirus and Isavirus (Wadell Harrach et al., 2011), display a similar genome structure of segmented, linear single-stranded RNA with negative polarity. Among the individual genera, the number of segments, the structure and number of glycoproteins as well as the host range differ.
Both influenza A and B viruses have eight genome segments and possess two membrane associated glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Whereas influenza A viruses can infect a variety of avian and mammalian species, including humans, influenza B viruses are restricted to humans. Influenza C viruses contain only seven segments and express no NA, but instead a protein with combined function of receptor-binding and sialidase activity, the hemagglutinin-esterase-fusion (HEF) protein (reviewed in: Krug and Fodor, 2013). Members of the Thogotovirus genus, Thogoto and Dhori viruses, harbour six and seven gene segments, respectively. Both viruses possess a non-influenza virus related protein (GP) which shares similarities with Baculovirus gp64 protein (Morse et al., 1992).
Influenza A viruses are further sub-classified based on HA and NA antigenicity. Until today, 17 HA (H1-H17) and ten NA subtypes (N1-N10) have been identified. The WHO Memorandum ("A revision of the system of nomenclature for influenza viruses: a WHO memorandum.," 1980) defined the full nomenclature for each new influenza isolate to include, in that order, virus type (A, B or C), the host of origin (except humans), the country of isolation, an individual strain number, and the year of isolation. For example, A/duck/Alberta/35/1976 (H1N1) defines an influenza A virus isolated

from a duck in Alberta in 1976.This virus belongs to the H1N1 subtype, 35 is the sequential number of the strain.

1.3 Influenza A virus morphology and genome structure

Influenza A virions have a spheroidal or filamentous shape with a diameter of 80 to 120 nm (**figure 1.1**). The subviral components can be subcategorized into three major structures: viral envelope, matrix, and the ribonucleoprotein (RNP) core (reviewed in Nayak et al., 2013).

The envelope is formed by a lipid bilayer, derived from the host cell plasma membrane during virus budding. The viral envelope incorporates three viral transmembrane proteins. The viral HA forms homotrimeric structures and represents the most abundant envelope protein (80%). The viral NA is embedded as a homotetramer into the envelope and with about 17% states the second highest membrane protein proportion. Each protein extends 10 to 12 nm from the membrane. A third transmembrane protein, M2, is incorporated into the viral membrane in minor quantities, forming a proton channel.

The matrix protein M1 covers the inner side of the lipid bilayer. M1 interacts with the cytoplasmic tails of the membrane-associated proteins HA, NA, and M2, and at the same time binds to each of the eight RNPs within the virion core.

Eight RNPs represent the large part of the virion core. Each RNP is formed by one of the eight different single-stranded RNA segments covered by several copies of the nucleoprotein (NP). A heterotrimeric polymerase complex consisting of PB1 (polymerase basic protein 1), PB2 (polymerase basic protein 2) and PA (polymerase acid protein) binds to a partially double-stranded panhandle structure formed by conserved 5’ and 3’ RNA termini of each segment. This results in a twisted rod-like structure that is folded back and coiled on itself. Recently, minor proportions of non-structural viral proteins were found to be present in the virion; these are NS1 and the nuclear export protein NEP/NS2 (reviewed in Nayak et al., 2013).

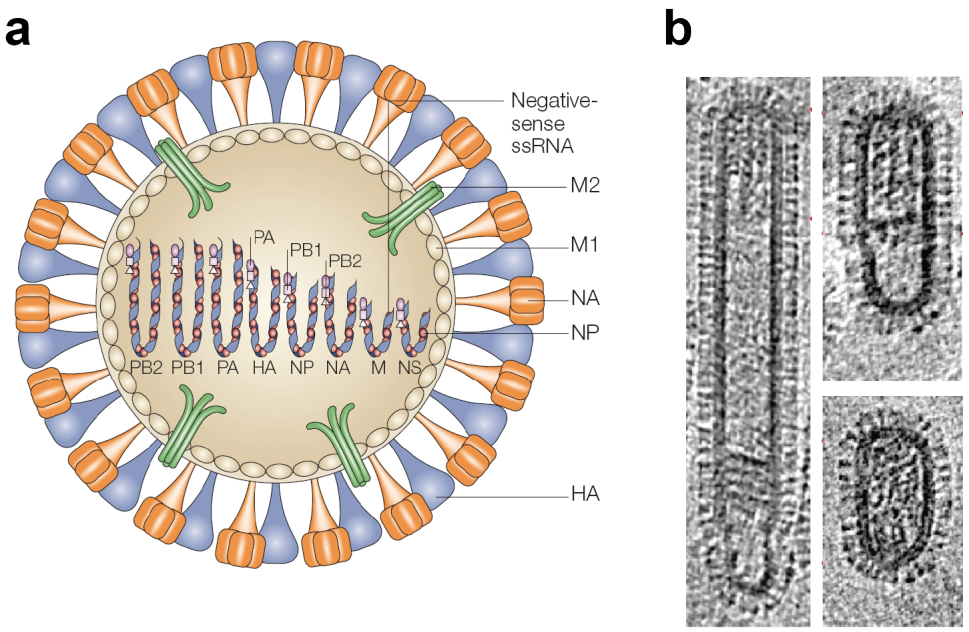

Figure 1.1: Influenza A virion. ***a*** *Schematic illustration; modified from Clancy, 2008.* ***b*** *Electron microscopic picture of filamentous and spherical virions (Calder et al., 2010).*

The influenza A genome consists of eight single-stranded RNA segments with a negative polarity (vRNA). Each segment codes for up to three viral proteins (**table 1**). The three largest segments encode the polymerase complex proteins PB2 (segment 1), PB1 (segment 2) and PA (segment 3). Some influenza virus strains encode the proapoptotic PB1-F2 on segment 2, which is synthesized using the +1 reading frame. Segments four to seven code for the structural proteins HA (segment 4), NP (segment 5), NA (segment 6), M1 (segment 7) and M2, a splice product of segment seven. The eighth segment encodes the nonstructural protein NS1 and the splice variant NEP/NS2. Coding regions of each segment are flanked by 3' and 5' non-coding regions. Each region can be separated into a highly conserved part at the ends of each segment, and variable segment-specific regions directly up- and downstream of the coding sequence.

Table 1: Overview of viral RNA segments and encoded proteins. *The table was modified from Cox et al., 2004; Vasin et al., 2014.*

Genome Segment	Length, nt[a]	Protein	Length, aa	Function
1	2341	**PB2**	759	Component of the viral RNA transcriptase complex; recognition of the 5'-capped host pre-mRNAs; affect host range and virulence
2	2341	**PB1**	757	Catalytic subunit of viral RNA transcriptase complex; RNA-dependent RNA polymerase; required for both replication and transcription
		PB1-F2	87	Influenza A virulence factor; induction of mitochondria-associated apoptosis; affects PB1 polymerase activity
		PB1 N40	718	N-terminal truncated form of PB1; maintenance of balance between PB1 and PB1-F2 expression
3	2233	**PA**	716	Component of viral transcriptase complex; Promoter binding; RNA endonuclease activity
		PA-X	252	Modulation of innate host response and viral virulence
		PA-N155	562	Function unknown; not essential
		PA-N182	535	Function unknown; not essential
4	1778	**HA**	566	Surface glycoprotein; receptor-binding; membrane fusion; major antigen
5	1565	**NP**	498	Major component of viral RNP complex; regulates nuclear cytoplasmic RNA transport
6	1413	**NA**	454	Surface glycoprotein; sialidase activity; facilitates virion release from cell surface by removal of sialic acids from infected cells
7	1027	**M1**	252	Forms matrix layer beneath viral envelope; multiple roles in infection and assembly; Involved in RNP nuclear export
		M2	97	Ion-channel activity; involved in RNP release; splice product of segment 7
		M42	99	Alternative splice product of segment 7; ion-channel activity; not-essential
8	890	**NS1**	230	Multifunctional non-structural protein; interferon antagonist; inhibits mRNA nucleus export; regulation of host and viral gene expression
		NS2 (NEP)	121	NS1 splice variant; structural component of viral particle; viral nuclear export protein
		NS3	174	NS1 splice variant; associated with mouse adaptation; function unknown

[a] Influenza A virus A/PR/8/1934 (H1N1)

1.4 Influenza A virus replication cycle

Viral replication is a complex process involving numerous viral and host cell factors (reviewed in: Krug and Fodor, 2013). HA-mediated virus adsorption to terminal sialic acid receptors attached to glycoproteins or lipids on the host cell surface initiates cell infection (**figure 1.2**). The viral sialidase NA helps to overcome the mucus barrier by cleavage of non-functional decoy receptors. Additionally, NA supports viral entry, facilitating elution from the cell surface in the course of a dynamic attachment-elution cycle during the entry process. Receptor-binding induces receptor-mediated endocytosis of the virion into clathrin-coated endocytotic vesicles. There is evidence that a minor fraction of virions is endocytosed using an alternative, clathrin-independent pathway (de Vries et al., 2011; Rossman et al., 2012).

The virus-containing endosomes mature to lysosomes and are acidulated by membrane associated H^+-ATPases. The viral M2 proton channel allows protonation of the virion core. This mediates dissociation of M1 from RNP-associated NP proteins, preparing the release of RNPs into the cytoplasm. By the time the endosomal pH drops below a threshold, ranging from pH 5.5 to 5.0, a conformational transition of the HA is triggered. As a result, the HA fusion peptide integrates into the endosomal membrane and facilitates fusion of the viral and endosomal membranes (described in detail in **1.5.2**). Consequently, vRNPs are released into the cytoplasm.

Unusual for negative-sense RNA viruses, transcription and replication of influenza A viruses take place in the nucleus. Two nuclear localization sequences (NLS) were identified in the NP protein. These motifs recruit alpha importins to RNPs and allow nuclear import using the classical import pathway (reviewed in Hutchinson and Fodor, 2013).

After nuclear import, primary transcription is initiated. A so far unknown mechanism of selective transcription restricts synthesis to primary transcript mRNA; namely, the polymerase genes, NP and NS1. At first, full length complementary RNA (cRNA) is synthesized along viral RNAs (vRNA). vRNAs contain 5' triphosphates, where the viral polymerase complex can initiate cRNA synthesis *de novo*. The cRNA is used as a template for further vRNA amplification and subsequent mRNA production. Various models were proposed for cRNA and vRNA synthesis involving several viral (NP, viral polymerase proteins, NS1, NEP, small viral RNAs) and host proteins (e.g. MCM, UAP65, tat-SF1, capped RNA primers and ribonucleoside triphosphates; reviewed in

Nagata et al., 2008; Resa-Infante et al., 2011). Nevertheless, the specific mechanism of vRNA and cRNA synthesis remains unknown. cRNA synthesis peaks early in infection, followed by vRNA synthesis starting two hours after infection. Together with vRNA synthesis, mRNA production increases within the first hours.

As the viral polymerase is not able to initiate *de novo* mRNA transcription, primers are excised from cellular pre-mRNAs. The process, called 'cap snatching', transfers cellular 5'-cap structures from mRNA to vRNAs. This process is initiated by vRNA binding to PB1. The binding prompts the association of 5'-cap structures of cellular mRNAs with PB2. After annealing to the 3' ends of the vRNA, the cellular primer is cut 10 to 13 nucleotides downstream of the 5'-cap by the endonuclease PA. The free 3' end is then used to prime further transcription performed by the viral RNA-dependent RNA-polymerase PB1. The transcription elongates until a uridine repetition of four to seven units is reached 16 to 20 nucleotides from the 5' end. Along the uridines a poly A tale is synthesized by polymerase stuttering. M2 and NS2 mRNAs are spliced by the cellular spliceosome. As splicing is incomplete, only 10 to 15% of the mRNAs are converted to M2 and NEP/NS2 mRNA, respectively (reviewed in: Krug and Fodor, 2013).

In a next step, the poly-adenylated mature mRNA is exported from the nucleus for subsequent translation. To facilitate export, viral polymerase complexes interact with the host polymerase II to recruit the transcription export (TREX) complex and NXF1/TAP (reviewed in: Krug and Fodor, 2013).

Localization sequences in viral mRNAs coding for membrane-associated proteins HA, NA and M2 direct them to ribosomes at the rough endoplasmic reticulum (rER). During translation, the growing polypeptide chains are translocated into the rER lumen and transmembrane domains are inserted into the rER membrane. During maturation the proteins travel along the Golgi- and trans-Golgi network and are modified post-translationally by glycosylation and palmitoylation (HA and M2). Additionally, HA precursor proteins (HA0) are cleaved into the two subunits HA1 and HA2 by intra- or extracellular host-proteases. Finally, the membrane-associated HA, NA and M2 proteins are transported to the cell membrane via the trans-Golgi-network (reviewed in: Krug and Fodor, 2013).

The viral mRNAs of the remaining proteins (M1, NP, PB1, PB2, PA, NS1 and NEP/NS2) are translated at free cytoplasmic ribosomes. After synthesis, these proteins are relocated to the nucleus. Within the nucleus, PB1, PB2, and PA condense to new

polymerase complexes. With increasing concentration of polymerase complexes, viral mRNA transcription is inhibited, whereas vRNA synthesis continues. During transcription, the nascent vRNA is encapsidated by NP proteins, which interact with viral polymerase complexes resulting in RNP formation (reviewed in: Krug and Fodor, 2013).

In late infection stages, newly assembled RNPs are exported from the nucleus. The matrix protein M1 directly binds RNPs and interacts with NEP/NS2. The nuclear export factor Crm1 is then recruited by nuclear export sequences (NES) in NEP/NS2 causing nuclear depletion of RNPs. In the host cell cytoplasm, RNPs interact with recycling endosomes through Rab11; a protein of the vesicular transport system. Endosome-associated RNPs are transported to the apical plasma membrane, making use of the microtubule network. A small proportion of RNPs are observed to migrate independently of Rab11 by diffusion or short range movements along actin filaments (reviewed in Hutchinson and Fodor, 2013).

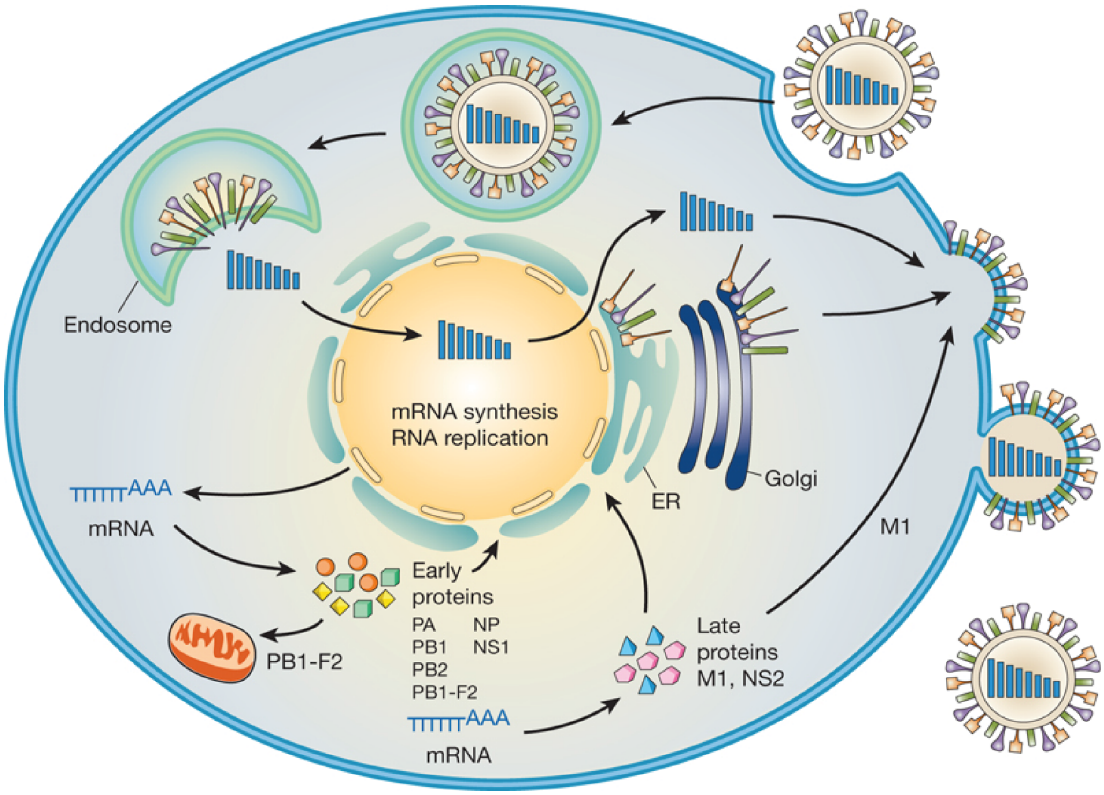

Figure 1.2: Schematic illustration of influenza A replication cycle. *The picture was modified from Neumann et al., 2009.*

At the cell membrane, assembly and subsequent budding is initiated at cholesterol- and sphingolipid-enriched membrane patches, so called lipid raft domains. Whereas HA

and NA are lipid raft associated, M2 accumulates at the rim of these domains (Leser and Lamb, 2005; Takeda et al., 2003). M1 proteins then bind to HA and NA cytoplasmic tails and interact with RNPs via NP binding at the same time. Packaging signals in the terminal regions of each segment lead to complex formation of specific segments (reviewed in Hutchinson et al., 2010).

The detailed mechanism of virus budding is still poorly understood. Individually expressed HA, NA and M1 proteins are found to alter membrane curvature forming virus like particles (VLPs). Thus, the currently proposed model suggests that HA and NA initiate the budding process. Binding to HA and NA cytoplasmic regions triggers M1 polymerization at the budding site, resulting in prolonged budding. At the budding neck, M2 facilitates membrane scission by curving the lipid bilayer (reviewed in Rossman and Lamb, 2011). The released virion may remain attached to the host cell through HA-bound sialic acids. To free budding virions, NA sialidase activity facilitates removal of sialic acids at the cell surface (reviewed in Rossman and Lamb, 2011).

1.5 Glycoproteins of influenza A virus

1.5.1 Hemagglutinin

The influenza A virus HA, a type I transmembrane protein, is encoded by the fourth gene segment, and the mature protein consists of 566 amino acids. According to structural and antigenic properties of the 17 HA subtypes, they cluster into two groups (Gamblin and Skehel, 2010): group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17) and group 2 (H3, H4, H7 H10, H14, H15).

The first HA structure was resolved using X-ray crystallography for bromelain-released HA ectodomain of A/Aichi/1968 (H3N2) (Wilson et al., 1981). A highly conserved fusion domain close to the viral envelope and a membrane distal globular head domain linked by a stalk region can be distinguished. The core of the fusion domain forms a 54 residue triple stranded coiled coil, which is flanked by spring loaded B loops, α-helices and membrane proximal regions (**figure 1.3a**).

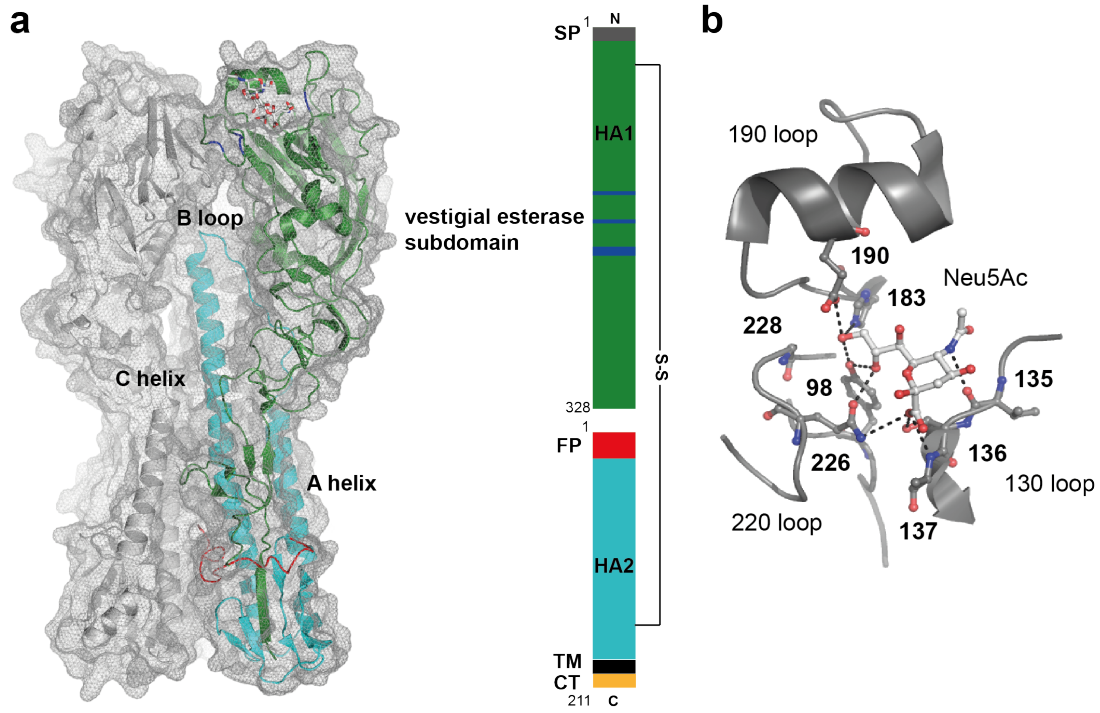

Figure 1.3: Influenza Hemagglutinin. a *Molecular and schematic model of a H1 HA trimer (2WRH, Protein Data Bank) with bound sialic acid receptor. Green, HA1; cyan, HA2; red, fusion protein; blue, receptor-binding site. SP, Signal peptide; TM, Transmembrane domain; CT, Cytoplasmic tail.* ***b*** *Receptor-binding site formed by 130 loop, 190 loop and 220 loop. Amino acids contacting sialic acid (Neu5Ac) are indicated. Hydrogen bonds are depicted as dotted lines.*

In HA0, the first 12 amino acids of the HA2 subunit form a prominent loop that is protease-accessible. After cleavage, the fusion peptide (amino acid 1 to 20 in HA2) is located in the protein close to the viral envelope fixed by hydrogen bonds. The fusion domain is kept in a high-energy conformation, partly stabilized by the globular head domain. The distal globular head harbours the receptor-binding pocket and a vestigial esterase subdomain. The head domain contains most of the major antigenic sites and is poorly conserved.

HA is synthesized as a HA0 precursor at the rER. During transportation through the trans-Golgi network it is glycosylated and palmitoylated at several positions. In the course of, post-translational processing, HA0 is proteolytically cleaved into two subunits (reviewed in Böttcher-Friebertshäuser et al., 2014; Steinhauer, 1999), HA1 and HA2, which remain connected via a single disulphide bridge (**figure 1.3a**). HA0 cleavage is essential for a low-pH triggered conformational change necessary for membrane fusion induction. Most avian and mammalian viruses harbour a single arginine, or in some cases a single lysine, at the cleavage site (reviewed in Böttcher-Friebertshäuser et al., 2014; Steinhauer, 1999). This monobasic cleavage site serves as

a substrate for trypsin-like proteases such as mini-plasmin (Murakami et al., 2001) or ectopic anionic trypsin I (Towatari et al., 2002). Recently, additional proteases in human airway epithelium, with similar cleavage motifs, were described to cleave HA0: TMPRSS2 (transmembrane protease serine S1 member 2), HAT (human airway trypsin-like) protease (Böttcher et al., 2006b) and TMPRSS4 (Chaipan et al., 2009). Expression of these proteases is restricted to defined tissues. This restricts Influenza A virus replication to the gastrointestinal tract in birds and the respiratory tract in mammals. In rare cases low pathogenic avian influenza (LPAI) viruses with a monobasic cleavage site acquire multiple repetitions of basic residues in the cleavage site by insertion or amino acid substitutions. These motifs (consensus sequence: R-X-R/K-R) are cleaved by ubiquitous available proteases such as furin or PC5 and PC6, resulting in systemic infections with high mortality rates. So far, these highly pathogenic avian influenza (HPAI) viruses arose from H5 and H7 subtypes only, with limited spread to humans.

During the viral replication cycle HA has two functions: i) virus adsorption to the cell via receptor-binding and ii) fusion of the viral and the endosomal membrane during cell entry.

1.5.1.1 Receptor-binding

Receptors for influenza A viruses are terminal sialyloligosaccharides (Sialic acids; Gottschalk, 1957) which are part of the cellular glycocalyx, composed of glycolipids, glycoproteins, proteoglycans and glycophospholipids (Varki and Sharon, 2009). Sialic acids represent the most diverse sugar on the cell surface with all sialic acid species sharing a nine-carbon backbone. The most common sialic acid species in mammalian cells are N-acetylneuraminic acids (Neu5Ac) and N-glycolylneuraminic acids (Neu5Gc) (Varki and Varki, 2007). Whereas Neu5Gc is not expressed in humans and birds, pigs present both Neu5Ac and Neu5Gc (Chou et al., 1998; Muchmore et al., 1998; Schauer et al., 2009; Walther et al., 2013). The horse trachea predominantly contains Neu5Gc (Suzuki et al., 2000).

Receptor-binding takes place at a topological depression in the globular head of HA1; the receptor-binding pocket (RBP) (**figure 1.3b**). The amino acids which contact

terminal sialic acids on oligosaccharides are conserved among subtypes (Nobusawa et al., 1991; Weis et al., 1988, 1990). The RBP bottom is formed by four conserved amino acids two tyrosine's (residue 98 and 195, H3 numbering here and following), one tryptophan (residue 153) and one histidine (residue 183). The edges of the depression are built by the '130-loop' (residues 133-138), the '190-helix' (residue 190-198) and the '220-loop' (residues 221-228) (reviewed in Skehel and Wiley, 2000). For sialic acid recognition mainly hydroxyl groups in C8 and C9 within the sialic acid are important (Kelm et al., 1992; Matrosovich et al., 1992).

Differences in the chemical linkage between sialic acids and the following sugar correlates with viral host species (Rogers et al., 1983; Rogers and D´Souza, 1989; Rogers and Paulson, 1983). Whereas avian and equine influenza viruses preferably bind to terminal sialyl-galactosyl residues linked by a α2,3 linkage (Neu5Acα2,3Gal), most mammalian viruses, including human and pig viruses, display strong binding to terminal α2,6-linked moieties (Neu5Acα2,6Gal; Connor et al., 1994; Gambaryan et al., 2005; Ito et al., 1998; Matrosovich et al., 2000, 1997; Nobusawa et al., 1991; Rogers and D´Souza, 1989) (**figure 1.4**).

The diverse viral receptor-binding preferences correlate with the availability of sialyloligosaccharides at the infection site. Studies on the receptor distribution in epithelial cells of duck intestines, the site of infection in aquatic birds, reveal a strong abundance of Neu5Acα2,3Gal but no Neu5Acα2,6Gal (Gambaryan et al., 2002, 2003; Pillai and Lee, 2010). Ciliated cells of the human airway epithelium show high levels of Neu5Acα2,3Gal surface expression. In contrast, non-ciliated cells possess mainly Neu5Acα2,6Gal sialic acid moieties and efficient replication in this cellular subset seems to be crucial for human infection. Type II pneumocytes express both Neu5Acα2,3Gal and Neu5Acα2,6Gal (Matrosovich et al., 2004). Thus a switch in the receptor-binding specificity is needed for the establishment of avian viruses in pigs and humans (reviewed in Baigent and Mccauley, 2003; Horimoto and Kawaoka, 2001; Matrosovich et al., 2000). In agreement with receptor availability, human and avian viruses differ in their cell tropism upon human infection. The respiratory epithelium of pigs was believed to contain both receptor types (Ito et al., 1998; Nelli et al., 2010; Scholtissek, 1990), leading to the common notion of pigs as 'mixing vessel' during human adaptation of avian viruses (Kida et al., 1994). However, current knowledge suggests that the receptor distribution in pigs is similar to the human respiratory tract (Van Poucke et al., 2010) and that pigs are not unique in the role as intermediate host.

Some avian species, e.g. chickens and common quail, are found to express both α2,3- and α2,6-linked sialic acids on respiratory and intestinal epithelia (Costa et al., 2012; Nelli et al., 2010; Trebbien et al., 2011).

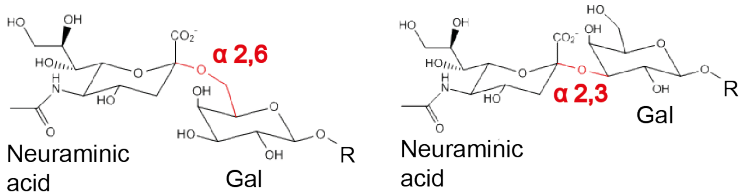

Figure 1.4: Structure of human (α2,6) and avian type (α2,3) sialic acid receptors. *Gal, galactose; R, penultimate oligosaccharide, usually ß1-3/4GlcNAc or ß1-3GalNAc.*

All pandemic viruses preferably bind to Neu5Acα2,6Gal-terminated sugars, although weak binding to avian type receptors was detected for some isolates (Rogers and D´Souza, 1989). Structural studies revealed that the switch of receptor preference is accomplished by amino acid substitutions which "widen" the RBP (Gamblin et al., 2004).

In human H2 and H3 viruses the two amino acid substitutions, Q226L and G228S (H3 numbering), determine a switch in receptor-binding preference from avian (α2,3 linkage) to human type (α2,6 linkage) receptors (Connor et al., 1994; Naeve et al., 1984; Rogers and Paulson, 1983). Additional mutations in close proximity (residues 136, 190 and 225; H3 numbering) modulate binding specificity and affinity (Martin et al., 1998; Matrosovich et al., 2000; Nobusawa et al., 2000). For human H1 viruses introduction of asparagine in residues 190 and 225 facilitates a switch in receptor-binding specificity (Glaser et al., 2005; Matrosovich et al., 2000).

Most avian viruses share general binding to terminal Neu5Aca2-3Gal, but differ in recognition of modified subterminal saccharides. Duck viruses from various subtypes (H1 to H5, H9 and H11) preferably recognize Neu5Acα2,3Gal connected by a ß1-3 linkage to the penultimate sugar, e.g. Neu5Acα2-3Galß1-3GlcNAc and Neu5Acα2-3Galß1-3GlcNAc. Additional sulfation of the subterminal GlcNAc residue at the 6-OH group does not influence binding. In contrast, fucosylation significantly reduces binding (Gambaryan et al., 2006, 2005, 2008).

Gull viruses of subtypes H4, H6, H13 and H14 show high binding avidity to fucosylated α2,3 sialyloligosaccharides (Neu5Acα2-3Galß1-4(Fuca1-3)GlcNAc; Neu5Acα2-3Galß1-3(Fuca1-3)GlcNAc), unaffected by the type of linkage to the subterminal sugar

(Gambaryan et al., 2005; Yamnikova et al., 2003). Terrestrial poultry H5, H7 and H9 viruses show most abundant binding to fucosylated and sulfated sialyloligosaccharides with ß1-4 connections between terminal and penultimate sugar residues; e.g. Neu5Acα2-3Galß1-4(6-O-HSO3)GlcNAc, Neu5Acα2-3Galß1-4(Fuca1-3)(6-O-HSO3)GlcNAc (Gambaryan et al., 2005, 2008, 2004). Accordingly, H5 and H9 viruses with 'duck-virus-like' and 'poultry-virus-like' binding properties were isolated from corresponding species (Gambaryan et al., 2005, 2008, 2004). Additionally, avian H5 and H7 HAs are found to have more glycosylation sites, which are associated with reduced binding affinity (Matrosovich et al., 1999).

Interestingly, some avian viruses show limited binding to human type receptors. For example, several quail H9N2 (Matrosovich et al., 2001; Saito et al., 2001) and a few Eurasian poultry H7 viruses (Belser et al., 2008; Gambaryan et al., 2008; Yang et al., 2010) were identified to recognize Neu5Acα2-6Gal. In consistence with binding specificities, chickens and quails possess both Neu5Acα2-3Gal- and Neu5Acα2-6Gal-terminated sialyloligosaccharides at the respiratory and intestinal epithelia (reviewed in Nicholls et al., 2008).

Human H7N9 isolates obtained in Southeast Asia during an outbreak in 2013 display binding to both human and avian type receptors (Ramos et al., 2013; van Riel et al., 2013; Watanabe et al., 2013; Zhou et al., 2013). As a consequence, they effectively attach to cells of the human upper respiratory tract as well as type I and type II pneumocytes in the lower respiratory tract (van Riel et al., 2013). The enhanced binding to α2,6-linked sialic acids is conferred by a leucine at residue 226, a substitution facilitates human type binding in H9N2 viruses, too. Potentially, binding was further enhanced by the introduction of valine at residue 186, which was reported to influence H7 HA receptor-binding (Gambaryan et al., 2012; Yang et al., 2013). Unlike human-adapted H3 viruses, none of the H7N9 viruses contain 228S.

1.5.1.2 Membrane fusion

The second function of HA during viral cell entry is to mediate fusion of the viral and endosomal membranes (reviewed in Russell, 2014).

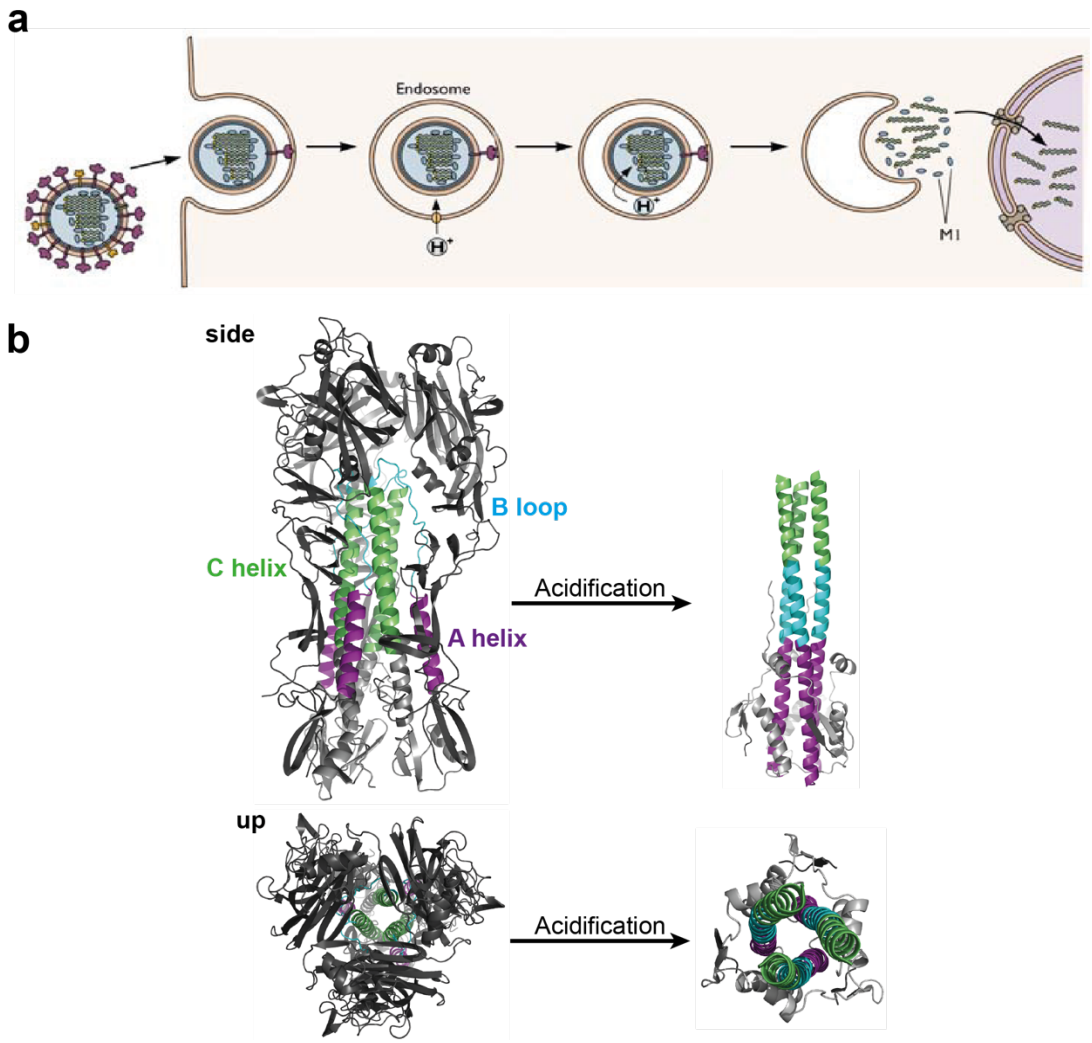

Figure 1.5: Illustration of HA-mediated membrane fusion. ***a*** *Schematic illustration of influenza A cell entry (http://www.virology.ws).* ***b*** *Structural rearrangement of HA during cell entry following acidification. The B loop (cyan) of the meta-stable neutral pH HA (2WRH, protein data bank) connecting the A (purple) and B helix (green) folds back into an alpha helix upon acidification (1HTM, protein data bank).*

HA-mediated receptor-binding initiates virion internalization into the host cell, by clathrin-mediated or clathrin-independent endocytosis (Lakadamyali et al., 2004; Matlin et al., 1981). Following internalization, virion-containing vesicles are transported through the endosomal pathway (**figure 1.5a**). The endocytosed material is exposed to pH 6.5 to 6.0 within the first 5 minutes. This early endosome is further acidulated by H^+-ATPases to pH 5.5 to 5.0 in late endosomes and up to pH 4.8 in lysosomes (Sun and Whittaker, 2013).

With decreasing pH, the M2 ion channels allow protonation of the virion core. As a result, the M1-RNP interaction is weakened, which assists RNP release later in infection (reviewed in Scott and Griffin, 2015).

At a threshold pH, the meta-stable HA undergoes a conformational transition, starting with distortion of the globular head and relaxation of the B loop (residues 56-75, H3 numbering) (**figure 1.5b**). Protons penetrate into the stalk region and induce a conformational change of the central helices (Xu and Wilson, 2011). Dissociation of the globular head allows the B loop to fold down, extending helix A (residues 76-126) through helix C (residues 38-55). This energetically favourable conformation induces the insertion of the fusion peptide into the target membrane. HA2 residues 106 to 112 shift from the coiled coil structure to a reversed turn, allowing the membrane-proximal region to zip up into the central coiled coil. This process brings the transmembrane domain in close proximity to the fusion peptide and results in hemifusion of the outer leaves of the membrane bilayers. The following fusion of the inner lipid layer creates a fusion pore, leading to vRNP release into cytoplasm. To create a fusion pore, at least six HAs have to act together (Dobay et al., 2011).

The threshold pH of fusion induction differs among virus strains and HA subtypes (Galloway et al., 2013; Scholtissek, 1985). To date, over 70 amino acid residues were identified to influence the pH optimum of fusion. They cluster in regions which dramatically change in secondary and tertiary structure during the conformational transition (reviewed in Russell, 2014). In addition, the enzymatic activity of NA was described to influence the pH of HA-mediated membrane fusion (Reed et al., 2010; Su et al., 2009) by a so far unknown mechanism.

Most of the pandemic viruses emerged during the twentieth century fuse at pH 5.2 to 5.0 (Galloway et al., 2013), leading to the classical point of view that membrane fusion occurs at the stage of the late endosome. In contrast, some swine viruses and the majority of tested H5N1 isolates induce the HA conformational transition between pH 4.6 and 6.0 (Galloway et al., 2013; Scholtissek, 1985), suggesting fusion induction at earlier stages during endosome maturation. In addition, viral adaptation to different cell lines alters pH optimum of membrane fusion (Lin et al., 1997; Murakami et al., 2012).

The energy barrier to induce transition of the meta-stable pre-fusion structure to the post-fusion conformation can also be overcome by heat, denaturant urea or low pH conditions (Carr et al., 1997; Ruigrok et al., 1986; Scholtissek, 1985). As the irreversible rearrangement of the protein abolishes HA receptor-binding capacity (Skehel and Wiley, 2000), the absence of a target membrane leads to viral inactivation. Therefore, viruses triggering the HA conformational transition at a higher pH are more

likely to lose infectivity in acidic environment during transmission and at the infection site prior infection (Reed et al., 2010).

For example, mutations in the stem region of an H9N2 HA affecting HA stability seem to be essential for transmission in ferrets (Sorrell et al., 2011). Recent studies show that HA stability also modulates transmission efficiency of *in vivo* adapted H5N1 viruses (Herfst et al., 2012; Imai et al., 2012; Linster et al., 2014). Thus, HA environmental stability is associated with the pH at which HA-mediated fusion is initiated, and there is accumulating evidence that HA stability influences inter- and intra-species transmission (discussed in detail in **1.7**). However, to date there is no formal proof that viral stability changes in the course of host switch. In addition, there is a gap in current knowledge as to whether the pH optimum of fusion and HA stability vary among viruses from different species.

1.5.2 Neuraminidase

The influenza A virus NA is a type II transmembrane glycoprotein, which is embedded in the viral membrane forming a tetramer complex stabilized by calcium (reviewed in Air, 2012). It consists of an amino-terminal signal domain, which is not cleaved and forms a highly conserved six amino acid cytoplasmatic tail, important for virion incorporation. The NA has a box-shaped head containing the catalytic centre (**figure 1.6a**) and major antigenic sites. The NA head is linked to the viral membrane by a stalk region of varying lengths, depending on subtype and host species (reviewed in Air, 2012). Within the host cell single monomers are synthesized at the rough endoplasmic reticulum and further processed in distal Golgi vesicles followed by transport to the cell membrane.

The catalytic side forms a deep pocket on the distal surface of the head domain and catalyses the cleavage of α-ketosidic linkages between terminal sialic acids and the adjacent sugar (Gottschalk, 1957). Within the catalytic centre nine highly conserved amino acids (**figure 1.6b**), of which six are basic (R118, R152, R224, R292, and R371), two acidic (E119 and E276) and two hydrophobic (W178 and I222), contact the sialic acid (Lentz et al., 1987).

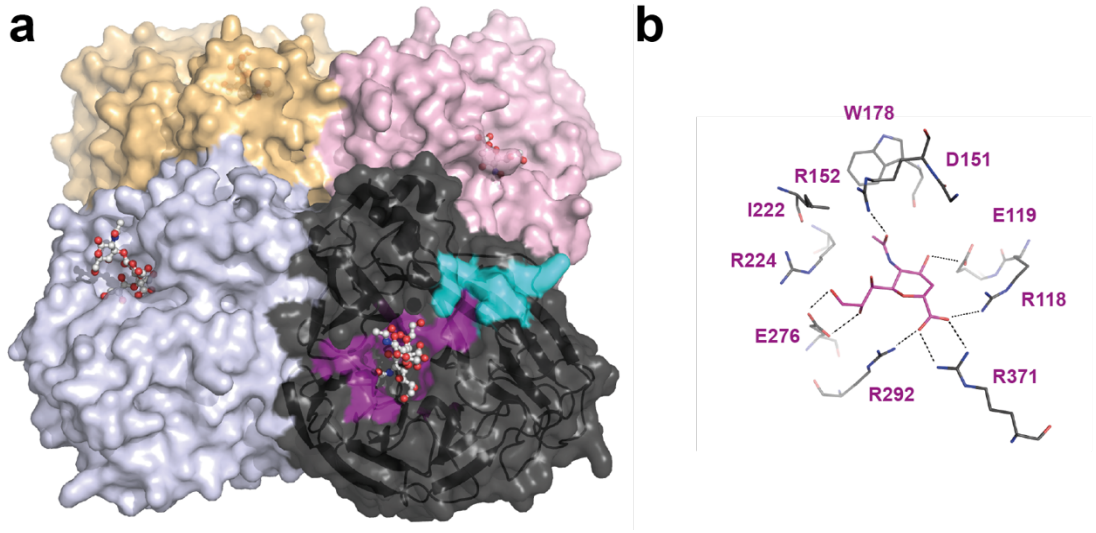

Figure 1.6: Influenza A virus neuraminidase. a *Structure of an N1 neuraminidase tetramer (2HTY, protein data bank) in complex with sialic acid. Catalytic centre is coloured in purple, hemadsorption site (HAD) in cyan.* ***b*** *Conserved amino acids forming the catalytic centre binding sialic acid. Hydrogen bonds are indicated as dotted lines.*

NA promotes viral entry and virion release from the cell by cleavage of terminal sialic acids. During cell entry NA cleaves soluble receptor analogues expressed in human mucus, preventing competitive inhibition of HA. After virion assembly and in the course of viral budding, NA depletes sialic acids from the cell membrane at the budding site. This process facilitates both virion detachment from the cell surface and prevention of virion agglutination after release (reviewed in Air, 2012).

Analogous to HA receptor-binding specificity NA recognizes different sialic acid substrates, with a preference depending on the host organism. Avian NA cleaves NeuAcα2,3Gal preferred over NeuAcα2,6Gal. In correspondence to receptor-binding a switch in the NA substrate preference from NeuAcα2,3Gal to NeuAcα2,6Gal was observed for human N2 NA after introduction into the human population (Baum and Paulson, 1991). The substrate specificity is mainly determined by glutamic acid in position 276 within the catalytic centre (Kobasa et al., 1999).

For many years, NA activity is believed to play a role in host range restriction (Hinshaw et al., 1983b). For example, NA from viruses isolated from land-based birds exhibits a stalk deletion which decreases catalytic activity (Banks et al., 2001; Bender et al., 1999; Matrosovich et al., 1999). This deletion in many cases is accompanied by changes in HA receptor-binding avidity (Baigent and McCauley, 2001; Matrosovich et al., 1999; Spackman et al., 2003) and most likely reflects adaptation to differences in sialic acid availability. The compensation of altered receptor-binding avidity by modification of

NA enzymatic activity illustrates that a balance between HA binding and NA activity is needed for efficient virus replication (reviewed in Hughes et al., 2000; Wagner et al., 2002). Additionally, the sialidase activity of NA supports infection inducing apoptosis by TGF-ß activation (Schultz-Cherry and Hinshaw, 1996).
Since NA activity is crucial for replication in humans (reviewed in Air, 2012), the NA represents a target for antiviral treatment. Sialidase inhibitors, such as Oseltamivir (Tamiflu^{TM}), inhibit NA activity in a competitive way by blocking the catalytic centre. NA inhibitors are widely used for influenza A virus treatment in humans. At the same time, a rapid occurrence of escape mutants harbouring a single point mutation (E199G) (Gubareva et al., 1997) can be observed.

1.6 Influenza A virus ecology

The natural reservoir of influenza A viruses are wild aquatic birds of the orders *Anseriformes* (geese, ducks and swans) and *Charadriiformes* (gulls and shorebirds) from which almost all subtype combinations of NA and HA could be isolated (reviewed in Fouchier and Guan, 2013). Occasionally, influenza viruses spread to other avian species, including domestic poultry such as chickens, turkeys and quail, or mammalian species (e.g. pigs, horses, several carnivores including dogs and cats as well as sea mammals and humans) (**figure 1.7**). In rare cases continued circulation leads to virus adaptation to the new host resulting in new host-specific lineages. The diversity of subtype combinations isolated from non-aquatic avian and mammalian species is limited (reviewed in Yoon et al., 2014). Thus, interspecies transmission seems to be restricted by several viral and host properties. For example, avian viruses replicate poorly in the porcine and human respiratory tract as receptor abundance differs between avian and mammalian hosts. However, this restriction is not complete as avian viruses are frequently isolated from other species.

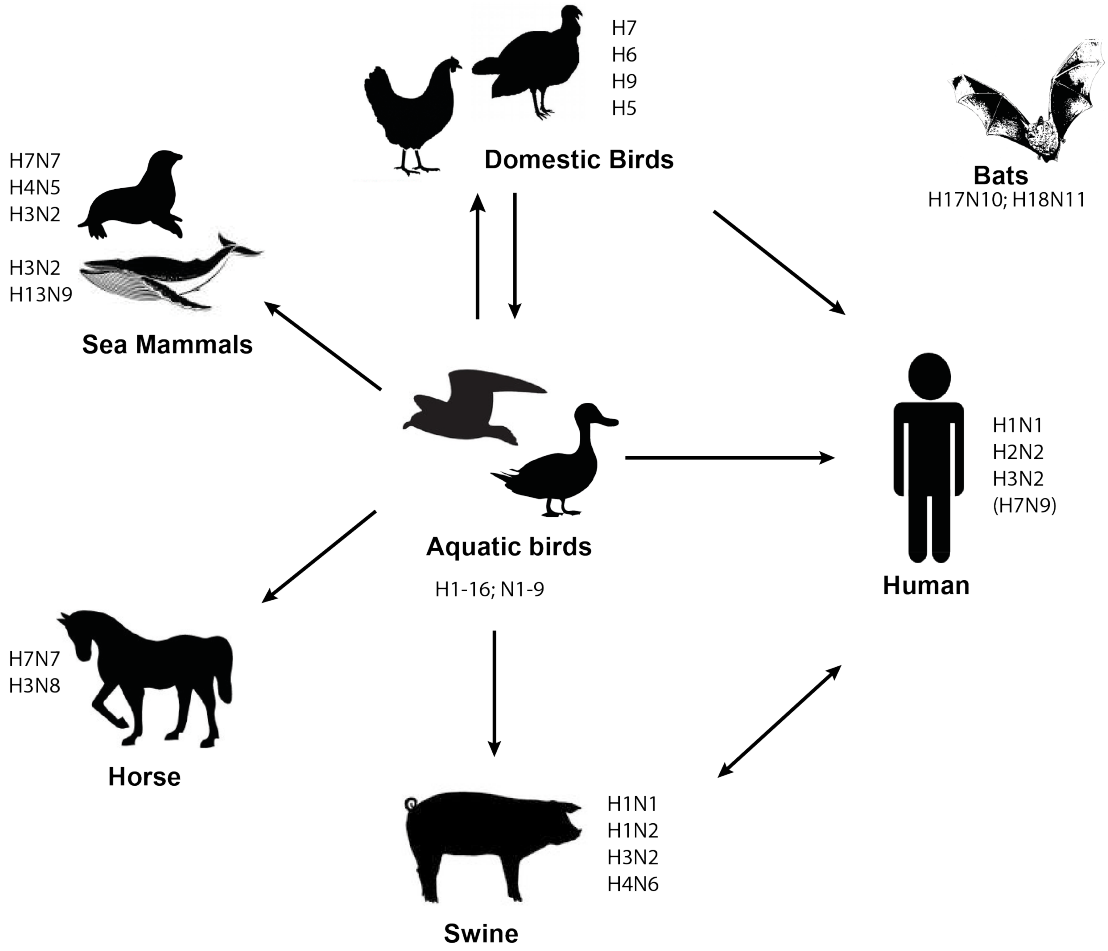

Figure 1.7: Influenza A virus ecology. *The illustration was modified from Webster et al., 1992.*

Currently, three endemic Influenza A virus subtypes are circulating in pigs, forming distinct geographically restricted lineages; H1N1, H3N2 and H1N2 (Vincent et al., 2014). Many of these viruses are descendants of human viruses. Although there are several reports of sporadic infections with avian viruses, only one circulating lineage, the so-called avian-like swine lineage, originated from an avian source. Over the last two decades, swine viruses were frequently isolated from humans (reviewed in Yoon et al., 2014) and turkeys (Olsen et al., 2003). Based on the avian and human receptor type distribution, pigs have been postulated as an intermediate host during the emergence of pandemic human strains as well (Ito et al., 1998; Scholtissek et al., 1983). Still, there is phylogenetic evidence indicating a genetic flow from man to pigs rather than the other way round (reviewed in Yoon et al., 2014).

Domestic birds are susceptible to numerous subtypes, normally presenting mild symptoms or no disease at all (reviewed in Franca and Brown, 2014). The majority of viruses isolated from poultry species belong to the subtypes H5, H6, H7 and H9 (reviewed in Neumann and Kawaoka, 2015). Viruses from the H5 and H7 lineages may

evolve into highly pathogenic viruses upon introduction into poultry by generation of a multiple basic amino acid motif in the HA cleavage site. This results in cleavage by ubiquitous proteases facilitating systematic spread and mortality rates up to 100% (reviewed in Franca and Brown, 2014).
Among terrestrial poultry quails are unique, as they support replication of a wide spectrum of influenza viruses. As the quail respiratory tract possesses both avian and human type receptors, quails may act as an intermediate host during human adaptation (Perez et al., 2003). However, no quail-to-human transmission has been described yet.

Influenza virus infections are common respiratory diseases in horses. In 1956, H7N7 viruses were found to circulate in the horse. After the occurrence of an H3N8 virus, first isolated in 1963, the H7N7 virus seemed to be eradicated step by step from the horse population (Webster et al., 1992). Today, H3N8 viruses circulate globally in horses, forming two geographically distinct lineages; American and European H3N8 viruses (reviewed in Yoon et al., 2014). The coincidence of widespread respiratory diseases in horses and man before 1900 suggests horses may have served as intermediated hosts in the past (reviewed in Cox et al., 2004).
Mammalian carnivores, such as cats and dogs, had not been considered as influenza virus hosts for a long time. Recent studies, however, describe two influenza subtypes circulating in dogs: H3N8 virus of avian origin and avian-like H3N2 viruses. Cats were found to be infected with viruses of avian (H7N3) and mammalian origin (human H2N2 and H3N2; seal H7N7; reviewed in Yoon et al., 2014).
Sporadically, influenza viruses are isolated from marine mammals as well. Between 1979 and 1980, outbreaks of H7N7 and H4N6 viruses resulted in a mass mortality among North American seals. Later, H4N6 and H3N3 viruses were isolated from tissue samples of stranded seals. In whales, viruses of the H13 subtype were detected at several occasions (reviewed in Yoon et al., 2014).
Even though bats harbour a variety of different virus species, so far they have not been considered as reservoir for influenza viruses. Recently, the genomes of two new influenza subtypes were amplified from bats; H17N10 and H18N11 (Tong et al., 2013).

The variety of influenza A hosts increases the risk of human infection. Within the massive increase in food demand and the growing population during the last century, the interface between influenza virus hosts is enlarged tremendously. For example,

backyard poultry farms and live poultry markets present optimal conditions for interspecies transmission and the exchange of viruses between waterfowl and terrestrial poultry. Additionally, the housing conditions of domesticated pigs of populations in a condensed area may facilitate rapid virus amplification. Therefore, domestic animals such as poultry and pigs which are in frequent contact with humans may act as potential intermediate hosts in the course of influenza virus transmission to man (Cauldwell et al., 2014).

1.6.1 Avian influenza

Wild birds are believed to state the natural reservoir of influenza A viruses (Olsen et al., 2006). For most of the viruses isolated from other hosts (humans, cats, seals, whales, pigs, horses and several bird species), an avian origin could be proven phylogenetically or antigenically (Li et al., 2004; Shortridge, 1992; Webster et al., 1992). This suggests avian species as the natural reservoir of influenza A viruses (Kawaoka et al., 1988; Webster et al., 1992).

Infection of avian species is mostly asymptomatic and restricted to the intestinal tract (Webster et al., 1978), but H5, H7 and H9 HPAI viruses can cause lethal systemic infection in poultry and aquatic birds.

The efficiency of virus replication after bird-to-bird transmission strongly depends on the species, virus strain and environmental factors (Alexander, 2007; Alexander et al., 1978; Narayan et al., 1969; Westbury et al., 1981). This may explain why out of the variety of influenza A virus subtypes mainly H5, H7 and H9 are circulating in domestic poultry species. The genetic similarity among aquatic bird and poultry viruses of these subtypes suggests continued gene exchange with ongoing cross-infections (Alexander, 2007)

In 1997 a H5N1 HPAI virus outbreak was detected on poultry markets in South East China. The virus origin was traced back to a progenitor circulating in geese one year earlier. In May 1997 the first human case of H5N1 HPAI virus infection was documented in Hong Kong. By December 18 cases were confirmed of which six died (Shortridge et al., 1998). Most cases were linked to direct poultry contact with limited human-to-human spread (Buxton Bridges et al., 2000; Li et al., 2004; Peiris et al., 2004; Subbarao et al., 1998). After eradication of this H5N1 virus from domestic poultry by

the culling of millions of animals, the virus continued to circulate undetectably in Asian poultry. Later, in 2003/04, a descendant of this virus re-emerged in South East Asia (reviewed in Alexander, 2007). Since then H5N1 viruses have been endemic in South East Asian poultry markets.

Avian H7 viruses separate into the North American and Eurasian lineage with little genetic exchange between both. Introduction of H7 viruses to domestic poultry was detected on several occasions in Asia, Europe and the American continent. The huge reservoir of H7 viruses in domestic animals (Gilbert et al., 2008; Woo et al., 2006) and sporadic human infections in Asia and Europe (Campitelli et al., 2004; Fouchier et al., 2004) raised public health concerns. In vitro studies demonstrate that several H7 viruses, currently circulating in Asian poultry, bind human-type receptors (Gambaryan et al., 2012), replicate in respiratory tissue cultures (Zhou et al., 2013), but show only limited airborne transmission between ferrets (Belser et al., 2008).

Besides H5 and H7 viruses, H9N2 viruses have caused several outbreaks in poultry in Germany, Italy, Ireland, USA, South Africa, Korea, China, Pakistan and the Middle East during the last 25 years (reviewed in Alexander, 2007). Interestingly, H9N2 viruses were detected in Asian swineherds, too. Additionally, binding to human type sialic acid receptors could be shown for some H9N2 viruses, raising concerns about possible future pandemics (Matrosovich et al., 2001). The potential capacity to infect humans was proven in March 1999 when H9N2 viruses were isolated at two independent occasions in Hong Kong from non-severe cases. For five additional patients an infection with H9N2 viruses was shown later that year in mainland China (Peiris et al., 1999).

1.6.2 Swine influenza

Influenza A viruses are the main reason for respiratory diseases in pigs with half of the herds in North America showing influenza-specific antibodies. Thus, influenza A viruses exhibit a vast reservoir in pig populations worldwide.

In 1919 a new respiratory disease in pigs was described in Iowa which was associated to the spread of the 1918 pandemic (Koen, 1919). The observed symptoms were similar to those in humans infected with Spanish flu, and therefore the disease was called ‘swine influenza’. In the 1930s the causing agent of the disease was identified as an

influenza virus (Shope, 1931). After genetic material of the Spanish flu was isolated, the swine influenza virus was demonstrated to be genetically related to the 1918 pandemic virus (Taubenberger et al., 1997). Descendants of this so-called **classical swine lineage** continued to circulate on the American continent and genetically and antigenically remain relatively stable.

In 1976, the North American swine flu spread to Europe (Nardelli et al., 1978; Pensaert et al., 1981). In 1979, novel H1N1 viruses were isolated from pigs (Pensaert et al., 1981), closely related to European H1N1 duck viruses (Krumbholz et al., 2014; Pensaert et al., 1981; Scholtissek et al., 1983). Descendant viruses of this avian-to-swine transmission became endemic in Europe and Asia, forming the so-called **Eurasian avian-like swine lineage** (EAsw) (Kuntz-Simon and Madec, 2009; Vincent et al., 2014) and completely replaced the North American lineage.

In 1995, a North American classical swine virus reassorted with a circulating seasonal human H3N2 virus and acquired human HA, NA and PB1 genes. This so-called double reassortant later obtained an avian PA and PB2 gene following another reassortment. The resulting **triple reassortant** (Zhou et al., 1999) spread over North America and continued to circulate after 1998 in parallel to the H1N1 classical swine viruses. Subsequent reassortment between both lineages resulted in two new genotypes; H1N1 and H1N2 (Karasin et al., 2002, 2000). These new lineages maintained the triple reassortant internal genes, involving avian (PB2 and PA), swine (M, NP and NS) and human (PB1) genes, while H1 and N1 were replaced (Vincent et al., 2008).

Today, avian-like swine viruses are endemic in Eurasia but not detectable in the North American region. Both the classical swine viruses as well as viruses evolved from the triple reassortant do not circulate in Europe or Asia but are widespread in North America. Descendants of the human-like H3N2 viruses co-circulate with the other lineages in pig populations worldwide.

Swine viruses are shown to be able to infect other domestic animals, for example turkeys (Hinshaw et al., 1983a), as well as humans but with limited capacity to spread between humans (Goldfield et al., 1977). Vice versa, pigs are susceptible to some avian viruses of different subtypes (Kida et al., 1994). Additionally, human viruses are frequently isolated from pigs, and most of the swine virus lineages are descendants of human viruses (reviewed in Nelson et al., 2015). This shows that pigs are susceptible to swine, avian and human viruses, raising the opportunity for genetic reassortment. The exchange of genetic material may result in the emergence of influenza viruses with

new antigenic properties. Introduction of such a virus into a human population with little or no pre-existing immune response may cause the next pandemic. However, other mammals, including humans, and some avian species, such as turkeys, can be infected with avian and swine viruses and serve as 'mixing vessels' as well (Hinshaw et al., 1983a; Myers et al., 2007; Smith et al., 2009). Nevertheless, close contact of domestic pigs and humans may constitute an interface for avian viruses to facilitate adaptation to humans.

1.6.3 Influenza in humans

Influenza viruses have circulated in humans at least since the 16th century causing both seasonal outbreaks and global pandemics. Pandemics typically originate from influenza viruses expressing an HA with antigenic properties which humans have no pre-existing immunity to.

The first well-documented pandemic in 1918, the so-called H1N1 **'Spanish flu'** claimed 20 to 50 million deaths worldwide. Not before the late 1990s, the genome sequence was determined from archival formalin-fixed tissue samples (Taubenberger et al., 1997) and later from Arctic permafrost samples. Subsequently the pandemic virus was reconstituted using reverse genetics (Tumpey et al., 2005). Genome sequencing data indicates that this H1N1 virus is closely related to classical swine viruses and suggests an avian origin for HA. So far the genetic basis for the high virulence and mortality is not fully understood but it seems that the HA (Kobasa et al., 2004; Pappas et al., 2008), the replication complex (Pappas et al., 2008; Tumpey et al., 2005), the NS1 (Geiss et al., 2002) and the PB1-F2 protein (McAuley et al., 2007) contributed to this phenotype. Surprisingly, none of the typical amino acid motifs related to high pathogenicity, such as a multi-basic cleavage site or lysine at position 627 in PB2, are present in the pandemic virus.

After the pandemic expired in 1919, the virus continued to circulate in humans. In 1957, the H1N1 virus acquired HA, NA and PB1 gene segments from an avian H2N2 virus by gene exchange, the so called reassortment (Kawaoka et al., 1989; Scholtissek et al., 1978). The resulting novel H2N2 virus caused the second pandemic of that century, the H2N2 '**Asian flu**', killing around one million people worldwide.

The H2N2 virus continued to circulate in the human population before it reassorted with an avian H3 virus in 1968. There, the human virus obtained new HA and PB1 gene segments of avian origin (Kawaoka et al., 1989; Scholtissek et al., 1978), resulting in the emergence of the H3N2 '**Hong Kong flu**'. It is speculated that the relatively mild course of this pandemic is a consequence of pre-existing N2 antibodies in humans retained from the previous pandemic (Schulman and Kilbourne, 1969; Viboud et al., 2005).

In February 2009, a novel H1N1 virus was isolated from influenza-infected patients in Mexico. Genome analysis suggests that this virus was originated from pigs after reassortment of a triple reassortant swine virus circulating in North America with a Eurasian avian-like swine virus which provided a new N1 NA and M gene segment (Garten et al., 2009). This swine-like H1N1 virus spread rapidly all over the world with mild clinical outcome. Subsequently, the pandemic H1N1 virus was reintroduced into the North American pig population. Continued reassortment with swine viruses resulted in multiple virus variants containing one or more gene segments of the pandemic virus, which may have the potential to infect humans. As an example, a swine-originated H3N2 virus acquired the pandemic M gene segment and was transmitted to humans in North America. Between 2011 and 2012, 201 confirmed cases were reported, out of which one was fatal. Additionally, only half of the cases are related to direct contact to pigs, suggesting the potential for human-to-human transmission (Epperson et al., 2013).

Human infections with avian viruses were reported for three additional subtypes (H5, H7 and H9). In most cases, these zoonotic infections are self-limiting with no sustained transmission among humans.

In 1997 a HPAI H5N1 virus infected 18 people in Hong Kong out of which six died. This was the first reported incidence where a purely avian influenza virus caused severe illness in humans. After re-emerging of descendants of this virus in 2003 a total of 607 human cases were reported until end 2012; more than half of them lethal (reviewed in (de Wit and Fouchier, 2008).

Avian H7N7 viruses are associated with sporadic human infection. One of the biggest outbreaks started in 2003 in the Netherlands spreading to neighbouring countries. In the process, an entirely avian virus was transmitted to humans. The majority of the recorded 89 cases only developed conjunctivitis (Fouchier et al., 2004). The worldwide circulation in wild and domestic avian species and the principal capacity of H7 viruses

to infect humans raised global health concerns. A recent outbreak in South East China where a newly evolved H7N9 virus infected 125 people between March and May of 2013 confirmed these concerns (Gao et al., 2013; Guan et al., 2013). 75% of the cases were linked to poultry contact and only little human-to-human transmission was reported in some family clusters (Gao et al., 2013).

H9N2 viruses have circulated in poultry since the mid 1980s and are frequently isolated from pigs. Between 1998 and 2003, Southeast Asia reported seven human cases in total with mild symptoms. Viruses isolated from patients showed mammalian-like characteristics; e.g. recognition of human-type receptors. This suggests that H9N2 viruses can infect humans and this raises concerns H9N2 viruses may cause the next pandemic (reviewed in Herfst and Fouchier, 2014).

1.7 Determinants of host range

The potential of influenza A viruses to infect new species relies on multiple viral and host genetic factors. Since viruses interact with a variety of host-cell factors at every stage of the replication cycle, incompatibilities may abolish viral replication and transmission. Crossing the species barrier and emerging in new recipient species involves several phases: First, the habitats of donor and recipient species need to overlap, at least temporary, to allow interspecies contact. Second, pathogen-host interactions in single individuals of the recipient species which allow replication and pathogen shedding need to be established. Third, intraspecies contact between hosts is needed and pathogen spread needs to be facilitated to persist in the new species (reviewed in Kuiken et al., 2011). Host restriction factors which limit influenza virus spread to new species may explain the sporadic occurrence of human infections. Typically, they result in dead-end infections with no sustained transmission.

One of the best-studied host restriction factors on cellular level is the availability of suitable receptors on cell surfaces at the infection site. Epithelial cells of the duck intestines predominantly express α2,3-linked sialic acids (Siaα2,3; Ito and Kawaoka, 2000). Cells of the human tracheal epithelium primary contain α2,6-linked sialic acids (Siaα2,6). Sialic acids with an α2,3 linkage are found on some alveolar cells in the human lower respiratory tract (Matrosovich et al., 2004; Nicholls et al., 2007; van Riel et al., 2006). Consequently, avian viruses favour Siaα2,3, while human adapted viruses

were found to predominantly bind Siaα2,6 (Matrosovich et al., 2000). As a result, human-to-human transmission is restricted for avian viruses and a switch in the receptor-binding specificity is essential to facilitate productive infection and transmission in mammals. HA of the H1 subtype was observed to alter binding specificity to Siaα2,6 recognition after replacement of glutamic acid in residue 190 by aspartic acid in humans and pigs (Gamblin et al., 2004; Glaser et al., 2005; Matrosovich et al., 2000; Rogers and D´Souza, 1989).

For HAs of the H2 and H3 subtypes the substitution of glutamine by leucine in residue 226 and glycine by serine in residue 228 accomplished transition of receptor-binding specificity from α2,3 (avian type) to α2,6-linked (human type) sialic acids (Connor et al., 1994). The same amino acids may facilitate a change in sialic acid recognition of H7 viruses. The HA of the human H7N9 virus from 2013 possesses the human type leucine in position 226 but the avian type glycine in 228; resulting in binding to both receptor types (Watanabe et al., 2013). This virus is able to spread via respiratory droplets between ferrets, whereas viruses with the avian signature do not transmit (Belser et al., 2008). However, transmission is less efficient than for human adapted viruses (Belser et al., 2013; Watanabe et al., 2013; Zhou et al., 2013). This is supported by only two reported family clusters during the 2013 outbreak (Gao et al., 2013). Additionally, some North American H7 viruses display increased binding to human type receptors (Gambaryan et al., 2012) and transmit via direct contact but not via droplet transmission between ferrets (Belser et al., 2013, 2008). This suggests that an alteration of receptor-binding specificity alone is not sufficient to establish sustained airborne transmission in ferrets (Maines et al., 2011).

Indeed, differences in the mode of transmission restrict influenza viruses from crossing the species barrier. Whereas for avian viruses waterborne transmission is typical, all known human adapted viruses spread via respiratory droplets. Airborne transmission seems to be essential for circulation in humans and viral spread by direct contact appears to play a minor role. Two studies investigated adaptive mutations in H5N1 viruses needed for the establishment of airborne transmission in ferrets, a model organism for human-to-human transmission (Herfst et al., 2012; Imai et al., 2012). Thus, previous studies showed that a switch in receptor-binding specificity is needed for airborne transmission of avian viruses (reviewed in Neumann and Kawaoka, 2015), mutations conferring human type binding were artificially introduced into both HAs prior adaptation (Q222L/G224S and N224K/Q226L, respectively). Both groups

continuously passaged avian H5N1 viruses in ferrets and identified a limited number of mutations conferring airborne transmissibility. Mutations in HA affected two functional properties apart from receptor-binding specificity: i) receptor-binding avidity and ii) HA stability. Both airborne transmissible H5N1 viruses lost the same N-glycosylation site (pos. 158-160) in the globular head during adaptation by different amino acid substitutions (N158D and T160A, respectively). HA glycosylation is known to affect antigenicity, receptor-binding specificity, and avidity (reviewed in Wright et al., 2013). In both studies the removal of the glycosylation results in increased binding to human type receptors (Imai et al., 2012; Linster et al., 2014).
Furthermore, both viruses acquired an additional mutation in the HA stalk region (T318I and H110Y, respectively), which is found to increase HA stability. The higher stability was essential to facilitate droplet transmission but sole introduction of these mutations into the original avian virus was not sufficient to confer transmissibility (Imai et al., 2012; Linster et al., 2014). In fact, more than 70 mutations in the HA of several subtypes (H1-H3, H5 and H7) were identified to affect HA stability and pH of fusion induction (reviewed in Russell, 2014), and some could be linked to transmission (Reed et al., 2009). DuBios and colleagues demonstrated that a higher pH optimum of HA-mediated fusion correlates with increased virulence in certain avian species (DuBois et al., 2011). In contrast, mutations increasing the pH of the HA conformational change attenuates H5N1 viruses in ducks (Reed et al., 2010), but enhances replication in mice (Zaraket et al., 2013a) and the upper respiratory tract of ferrets (Zaraket et al., 2013b). Thus, HA stability and the pH optimum of membrane fusion may be critical to facilitate virus replication and transmission and changes are needed for establishment of new lineages. Until today, differences in membrane fusion activity and HA stability between species and alterations during interspecies transmission are poorly investigated.

Viral NA may also contribute to efficient replication and transmission. The abundance of viral receptors on target tissues differs among species. As a consequence, HA receptor-binding avidity may change during host switch. Alteration of receptor-binding properties after reassortment or transmission to new hosts may result in an imbalance of binding avidity and NA sialidase activity. Since an optimal balance between HA and NA features is essential for efficient viral replication and transmission (Lakdawala et al., 2011; Yen et al., 2011), an equilibrium has to be re-established upon transmission to novel host species. Changes in HA binding avidity can compensate for altered NA

activity and vice versa (Baigent and McCauley, 2001; Mitnaul et al., 2000; Wagner et al., 2002). For example, bird-to-chicken transmission frequently results in an NA stalk deletion (Banks et al., 2001; Hossain et al., 2008). The deletion enhances viral replication and pathogenicity in domestic poultry (Munier et al., 2010; Sorrell et al., 2010), and reduces virus release from the cell surface (Castrucci and Kawaoka, 1993; Matrosovich et al., 1999). In ferrets, an NA stalk deletion abolishes respiratory droplet transmission (Blumenkrantz et al., 2013).

Avian polymerases show a low replication efficiency in mammals, making an increase in polymerase activity necessary for host adaptation (Naffakh et al., 2008). PB2 represents the main determinant for host range and virulence among the three proteins of the polymerase complex PA, PB1 and PB2. The most prominent adaptive mutation in PB2 is E627K (Subbarao et al., 1993). Glutamic acid (E) is found in all avian isolates and restricts replication in mammals at 33°C; the temperature of the mammalian upper respiratory tract (Hatta et al., 2007). Lysine (K) is associated with mammalian adaptation and confers increased pathogenicity in mice as well as transmission in pigs and ferrets (Chen et al., 2007; Gao et al., 2013; Hatta et al., 2001; Shinya et al., 2004; Steel et al., 2009). The mammalian signature (K) is present in some human pandemic isolates (1957 and 1968) (Scholtissek et al., 1978), and is frequently selected during replication of avian viruses in humans and terrestrial poultry (reviewed in Wright et al., 2013). Moreover, 627K was detected in most of the human isolates of the human H7N9 virus in 2013 (Gao et al., 2013). It was shown that the mutation E627K partially compensates for decreased replication efficiency at lower temperatures within the human respiratory tract (33°C) compared to avian intestines (41°C) (Hatta et al., 2007; Scull et al., 2009). Hypothetically, this is facilitated by stabilizing the interaction of polymerase complex proteins PB1, PB2 and PA (Weber et al., 2015). A lack of 627K can be compensated by a basic residue in position 591 (Mehle and Doudna, 2009).

Other adaptive mutations in PB2 were described to favour replication in mammals. For example, the exchange of aspartic acid by asparagine in position 701 increases the virulence of avian viruses in mammals (Gabriel et al., 2005). In mammalian cells an increased replication efficiency was conferred by a T271A substitution (Bussey et al., 2010). The influence of the polymerase adaptation on transmission was shown for the 1918 pandemic. Whereas the HA and NA derived from the 1918 pandemic are not able to facilitate respiratory droplet transmission on their own, the addition of the pandemic PB2 resulted in airborne transmission between ferrets (Van Hoeven et al., 2009).

Other host-specific genetic signatures involved in host switch were identified within all polymerase subunits as well as the NP. Indeed, NP is described to interact with importin α (Gabriel et al., 2011; O'Neill et al., 1995) and is involved in the suppression of interferon (IFN) induction (Mänz et al., 2013).

The efficiency to prevent and control the cellular IFN response is one important factor determining host range. Virus driven IFN type I and II induction results in the expression of hundreds of antiviral genes capable to interfere with viral replication. The main IFN antagonist produced by influenza A viruses is the non-structural protein NS1. NS1 is able to block IFN induction by abolishing activation of retinoic acid-inducible gene 1 (RIG-I) as well as inhibiting the expression of other antiviral genes (Hale et al., 2008; Marazzi et al., 2012).

As the present study is focused on the role of HA membrane fusion activity as host restriction factor during interspecies transmission, other host range determinants are not investigated.

Aim of the study

In the course of interspecies transmission, influenza viruses undergo rapid evolution in response to novel selective pressures within the new host. Typically, interspecies transmission is restricted to individuals with no or limited spread. In rare cases these viruses acquire the ability to transmit and establish stable lineages in new species. The underlying host range restriction mechanisms and adaptive changes required for viral emergences in new host species are not fully understood.

The influenza HA possesses the major host specificity by determining receptor-binding specificity. Differences in the availability of avian (α2,3) and human type (α2,6) receptors at the infection site define host range and restrict interspecies transmission. Recently, the HA-mediated membrane fusion activity was suggested to contribute to interspecies transmission (Herfst et al., 2012; Imai et al., 2012). Nevertheless, knowledge about differences of pH stability and fusion in birds and mammalian species is limited. To address this question, this work is focused on the role of viral membrane fusion activity and HA stability during interspecies transmission using several experimental models.

First, adaptation of an avian influenza virus to pigs in Europe in the 1970s during the emergence of the avian-like swine lineage was studied. Second, an avian H1N1 virus experimentally adapted to pigs was characterised regarding receptor-binding specificity, fusion activity, and replication in human airway epithelial cells. Third, differences in fusion and HA stability between different bird species were investigated, comparing closely related duck and poultry viruses of the same HA subtype (H7). Fourth, the influence of stability and fusion on the emergence of pandemic viruses was studied with particular focus on the pandemic of 1968. Additionally, fusion activity of the novel H7N9 virus isolated from humans was examined.

2 Materials

2.1 Chemicals

Agarose Seakem®	Cambrex Bio Science, Rockland
Ampicilin (sodium salt)	Sigma-Aldrich, Steinheim; Germany
Avicel	FMC Biopolymer
Bacto-Agar	BD Biosciences, Heidelberg; Germany
Boric acid	Riedel-de Haen, Seelze; Germany
Bromphenol blue (sodium salt)	Roth, Karlsruhe; Germany
Calcium chloride ($CaCl_2$)	Merck, Darmstadt; Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Steinheim; Germany
Ethanol (EtOH)	Roth, Karlsruhe; Germany
Ethidium bromide (EtBr)	Roth, Karlsruhe; Germany
Ethylenediaminetetraacetic acid (EDTA)	Roth, Karlsruhe; Germany
Glycerol	Roth, Karlsruhe; Germany
Horse serum	Sigma-Aldrich, Steinheim; Germany
Hydrochloric acid (HCl)	Merck, Darmstadt; Germany
Hydrogen peroxide (H_2O_2)	Sigma-Aldrich, Steinheim; Germany
Magnesium chloride ($MgCl_2$)	Merck, Darmstadt; Germany
Monopotassium phosphate (KH_2PO_4)	Merck, Darmstadt; Germany
Monosodium phosphate (NaH_2PO_4)	Merck, Darmstadt; Germany
Paraformaldehyde (PFA)	Roth, Karlsruhe; Germany
Peptone	Merck, Darmstadt; Germany
Polyethylene glycol (PEG 4000)	Sigma-Aldrich, Steinheim; Germany
Sodium acetate (NaAc)	Merck, Darmstadt; Germany
Sodium chloride (NaCl)	Roth, Karlsruhe; Germany
Sodium hydroxide (NaOH)	Sigma-Aldrich, Steinheim; Germany
Tris(hydroxymethyl)aminomethane (Tris)	Acros Organics, Geel, Belgium
Triton X-100	Serva, Heidelberg; Germany
True Blue™ peroxidase substrate	KPL; USA
Tween 80	Sigma-Aldrich, Steinheim; Germany
Yeast extract	Merck, Darmstadt; Germany

2.2 Consumables

Cell culture flasks (25, 75 and 175 cm2)	Greiner, Frickenhausen; Germany
Cell culture plates (6, 12, 24 and 96 well)	Greiner, Frickenhausen; Germany
Cell scraper,	Greiner, Frickenhausen; Germany
Combi-Tips Ritips	Ritter, Schwabmünchen; Germany
Cryo-tubes (2 ml)	Corning; Netherlands
Eppendorf reaction tubes (1.5 and2 ml)	Eppendorf, Hamburg; Germany
Micro-tubes, screw capped (0.5, 1.5 and 2 ml)	Sarstedt, Nürnbrecht; Germany
Nunc-Immuno 96-well microtiter plates	Thermo Scientific, Langenselbold; Germany
Parafilm	Pechiney Plastic, Neenah
PCR reaction tubes (0.2 ml)	Biozyme, Hess. Oldendorf; Germany
Plastic pipettes (2, 5 and 10 ml)	Greiner, Frickenhausen; Germany
Polypropylene reaction tubes (15 and 50 ml)	Greiner, Frickenhausen; Germany
PP-Test tubes (5 ml)	Greiner, Frickenhausen; Germany
Scalpel	PfM AG, Colone; Germany
Single-use pipette tips	B Braun, Melsungen, Germany
TipOne pipette tips	Starlab, Ahrensburg; Germany

2.3 Commercial kits

Dual Luciferase® Reporter Assay System	Promega, Mannheim; Germany
HiSpeed Plasmid Maxi Kit	Qiagen, Hilden; Germany
One-Step RT-PCR Kit	Qiagen, Hilden; Germany
peqGold Plasmid Miniprep Kit I	PeqLab, Erlangen; Germany
QIAamp® viral RNA Mini Kit	Qiagen, Hilden; Germany
QIAquick® Gel Extraction Kit	Qiagen, Hilden; Germany
QIAquick® PCR Purification Kit	Qiagen, Hilden; Germany
QuikChange™ Site-Directed Mutagenesis Kit	Stratagene, Basel; Switzerland
RNeasy® Mini Kit	Qiagen, Hilden; Germany

2.4 Laboratory equipment

Eppendorf table centrifuge 5424	Eppendorf, Hamburg; Germany
Eppendorf research pipette (1-10 µl; 10-100 µl; 100-1,000 µl)	Eppendorf, Hamburg; Germany
GelDoc 2000	Biorad, Richmond; USA
Hera Cell 150 Incubator	Heraeus Instruments, Hanau; Germany
Hera Safe Biosafety cabinet	Heraeus Instruments, Hanau; Germany
Light microscope Optech Mod. 1B	Exacta + Optech GmbH, München; Germany
Luminometer Centro LB 960	Berthold Technologies, Bad Wildbach; Germany
Magnetic stirrer MR 2000	Heidolph, Schwabach; Germany
Microwave	Bosch
Multifuge 3S-R	Heraeus Instruments, Hanau; Germany
NanoDrop 1000	PeqLab, Erlangen; Germany
Optima™ L-100K Ultracentrifuge	Beckman Coulter, Krefeld; Germany
Precision scale	Sartorius, Göttingen; Germany
Shaker	Kreutz, Reiskirchen; Germany
Spectrafuge™ Mini-Centrifuge	Labnet International, Woodbridge; USA
Thermal Cycler 2720	Applied Biosystems, Darmstadt; Germany
Thermo block Dri Block DB-A2	Techne, Staffordshire; UK
Water bath 1004	GFL, Burgwedel; Germany

2.5 Buffer and solutions

Ammonium chloride solution (NH_4Cl; 1 M)	NH_4Cl	2.67 g
	dH_2O	50 ml
Ampicillin stock solution	Ampicillin	100 mg
	dH_2O	ad 1 l
ELISA buffer	Horse serum	10%
	Tween 80	1%
	in PBS^{def}	

Erythrocyte suspension (1%)	chicken blood	1 ml
	PBS^{def}	ad 100 ml
DNA sample buffer (5x)	Glycerol	3 ml
	Bromphenol blue	0.025 g
	Xylencyanole	0.025 g
	dH_2O	ad 10 ml
PBS^{++} (Phosphate buffered saline)	NaCl	8.00 g
	KCl	0.20 g
	Na_2HPO_4	1.15 g
	KH_2PO_4	0.20 g
	$MgCl_2$	0.10 g
	$CaCl_2$	0.13 g
	dH_2O	ad 1 l
PBS^{def} (Phosphate buffered saline)	see PBS^{++}, without $MgCl_2$ and $CaCl_2$	
Permeabilization buffer	Triton X-100	0.3 %
	Glycine	20 mM
	In PBS^{def}	
Reaction buffer	Tween 80	0.02 %
	BSA-NA	0.1 %
	Neuraminidase inhibitior in PBS^{def}	1 µM
Sodium acetate buffer pH 5.5 (3 M)	$C_2H_3NaO_2$	24.61 g
	dH_2O	Ad 100 ml
	pH adjusted:	Acetic acid

TBE buffer (10x)	Tris	108 g
	Boric acid	55 g
	0.5 M EDTA, pH 8.0	4 ml
	dH_2O	ad 1l
TSS buffer	PEG	10 g
(transformation and storage solution)	DMSO	5 ml
	1 M $MgCl_2$ pH 6.5	5 ml
	LB-Medium	85 ml
Washing buffer	Tween 80	1 ml
	PBS^{def}	ad 1 l

2.6 Nucleotides

Cloning Primers	5' → 3'
BM-HA-F	TATTCGTCTCAGGGAGCAAAAGCAGGGG
BM-HA-R	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT
BM-NA-for	TATTCGTCTCAGGGAGCAAAAGCAGGAGT
BM-NA-rev	ATATCGTCTCGTATTAGTAGAAACAAGGAGTTTTTT

Sequencing Primers	5' → 3'
H1dk900fwd	GTGACACAAAGTGCCAAACC
H1dk960-rev	TTGGGCATTCTCCAATAGTG
pCAGGS-46-F	ACGTGCTGGTTATTGTGC
pCAGGS-46-R	ATAATTTTTGGCAGAGGG
H3-F668	AGCATCAGGGAGAGTCAG
H3-R739	GGTCTGGACCCGATATTC
N1-824-R	AGGAGCATTCAACTCGACTG
N1-610-F	GGGCAGTGGCTGTATTGAAA

Mutagenesis Primers

QC-N-N416D-F	GGCAAGGAATTCAACGACCTAGAAAGAAGAATTGAG
QC-N-N416D-R	CTCAATTCTTCTTTCTAGGTCGTTGAATTCCTTGCC
QC-N-R419K-F	ATTCAACAATCTAGAAAAAAGAATTGAGAATTTGAATA
QC-N-R419K-R	TATTCAAATTCTCAATTCTTTTTTCTAGATTGTTGAAT
QC-N-T393S-R	GAGTTCACCTTGTTACTGATTCCGTCGATTGCA
QC-N-T393S-F	TGCAATCGACGGAATCAGTAACAAGGTGAACTC
QC-N-S457F-F	CTAGATTTCCATGACTTCAATGTGAGAAATTTG
QC-N-S457F-R	CAAATTTCTCACATTGAAGTCATGGAAATCTAG

2.7 Enzymes

Restriction Enzymes

*Dpn*I	5'…GA^{m6}|TC…3'	Fermentas, St. Leon-Rot; Germany
*Esp*3I (*Bsm*BI)	5'…CGTCTC(N)$_1$|…3'	Thermo Scientific, St. Leon-Rot; Germany

Other Enzymes

Dnase I	Thermo Scientific, St. Leon-Rot; Gemany
Pfu DNA-Polymerase	Thermo Scientific, St. Leon-Rot; Gemany
Phusion® High Fidelity DNA-Polymerase	NEB, Frankfurt a. M.; Gemany
RevertAiD H Minus M-Mulv Reverse Transcriptase	Thermo Scientific, St. Leon-Rot; Gemany
RiboLock™ RNase Inhibitor	Thermo Scientific, St. Leon-Rot; Gemany
Shrimp Alkaline Phosphatase	Fermentas, St. Leon-Rot; Gemany
T4 DNA-Ligase	Fermentas, St. Leon-Rot; Gemany
Vibrio cholerae sialidase	Sigma-Aldrich, Steinheim; Germany

2.8 Antibodies

α-Influenza A NP (mouse)	Centers for Disease Control and Prevention, Atlanta; USA
α-mouse IgG (rabbit), HRP conjugated	DAKO; Denmark
α-H1 IgG (rabbit)	Provided by Markus Eickmann, Marburg; Germany
Goat α-rabbit, Alexa Fluor® 488 dye conjugated	Thermo Scientific, Darmstadt; Germany

2.9 Cell culture

Prokaryotic cells

Escherichia coli, XL1-blue strain Genotype: recA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB laclqZΔM15 Tn10 (Tetr)]	Stratagene, La Jolla; USA

Media for prokaryotic cells

LB-Medium	Yeast extract	5 g
	Peptone	10 g
	NaCl	5 g
	dH_2O	1l
LB-Agar	1.5 % Bacto-Agar in LB-Medium	

Addition of 50 µg/ml Ampicillin after autoclaving
(Temperature below 50°C)

Eukaryotic cells

Name	Organism	Type
BHK-T7	*Mesocricetus auratus*, hamster, Syrian golden	baby hamster kidney fibroblasts; transient expression of T7-Polymerase
HEK293	*Homo sapiens*, human	kidney, embryonic
HeLa	*Homo sapiens*, human	cervical cancer
MDCK	*Canis familiaris*, cocker spaniel	kidney

Media for eukaryotic cells

Growth medium	1x DMEM	500 ml
	FCS	50 ml
	L-Glutamine [200 mM]	5.5 ml
	Penicillin/Streptomycin [5000 U/ml/5000 µg/ml]	5.5 ml
Infection medium	1x DMEM	500 ml
	BSA (30%)	0.1 %
	L-Glutamine [200 mM]	5 ml
	Penicillin/Streptomycin [5000 U/ml/5000 µg/ml]	5 ml
2x MEM infection medium	2x MEM	250 ml
	BSA (30%)	0.2%
	Penicillin/Streptomycin [5000 U/ml/5000 µg/ml]	5 ml
Overlay Medium	2x MEM infection medium mixed with Avicel solution	1:1

Supplements for eukaryotic cells

Autoclaved Avicel solution (2.5% (w/v) in dH_2O)	Institute of Virology, Marburg; Germany
Bovine serum albumin (BSA; 30%)	PAA, Linz; Austria
Dulbecco's modified Eagle's medium (DMEM)	Gibco BRL, Eggenstein; Germany
Fetal calf serum (FCS)	Gibco BRL, Eggenstein; Germany
L-Glutamine [200 mM], 100x	Gibco BRL, Eggenstein; Germany
Lipofectamine™ 2000	Invitrogen, Karlsruhe; Germany
Minimum essential medium (2xMEM)	Gibco BRL, Eggenstein; Germany
OptiMEM	Gibco BRL, Eggenstein; Germany
Penicillin/Streptomycin [5000 U/ml, 5000 µg/ml]	Gibco BRL, Eggenstein; Germany
Sodium bicarbonate solution, 7.5 %	Sigma-Aldrich, Steinheim; Germany
Trypsin EDTA solution, 0.05%	Gibco BRL, Eggenstein; Germany
Trypsin, tosyl phenylalanyl chloromethyl ketone (TPCK)-treated	Worthington, Lakewood; USA

2.10 Plasmids

Plasmid	Insert	Origin
pHW2000	Bi-directional expression plasmid Contains human RNA-Polymerase I promoter and RNA-Polymerase dependent cytomegalovirus (CMV) promoter This plasmid was used for generation of recombinant viruses (Hoffman et al., 2000; Hoffmann et al., 2001) and site directed mutagenesis	Provided by Erich Hoffmann and Robert Webster, St.Jude Children's Research Hospital, Memphis, TN, USA
pHW2000-HAwt	HA of A/duck/Bavaria/1/1977[a]	Dr Jürgen Stech; FLI Riems, Germany
pHW2000-HA49	HA of A/duck/Bavaria/1/1977; T492S	Jan Baumann
pHW2000-HA72	HA of A/duck/Bavaria/1/1977; N722D	Jan Baumann

Plasmid	Insert	Origin
pHW2000-HA113	HA of A/duck/Bavaria/1/1977; S1132F	Jan Baumann
pHW2000-HA-2009	HA of A/Hamburg/05/2009[b]	Jan Baumann
pHW2000-HA-Marseille	HA of A/swine/Marseille/2260/1980[c]	Jan Baumann
pCAGGS	Eucaryotic expression vector for mammalian cells; contains CMV-Promotor	Dr Mikhail Matrosovich
pCAGGS-HAwt	HA of A/duck/Bavaria/1/1977	Jan Baumann
pCAGGS-HA49	HA of A/duck/Bavaria/1/1977; T492S	Jan Baumann
pCAGGS-HA72	HA of A/duck/Bavaria/1/1977; N722D	Jan Baumann
pCAGGS-HA75	HA of A/duck/Bavaria/1/1977; R752K	Jan Baumann
pCAGGS-HA113	HA of A/duck/Bavaria/1/1977; S1132F	Jan Baumann
pCAGGS-HA-1918	HA of A/Brevig Mission/1/1918[d]	Dr Mikhail Matrosovich
pCAGGS-HA-1957	HA of A/Singapore/1/1957[e]	Jan Baumann
pCAGGS-HA-1968	HA of A/HK/1/1968[f]	Dr Tatyana Matrosovich
pCAGGS-HA-H7N9	HA of A/Shanghai/1/2013[g]	Genescript™
pCAGGS-HA-H7N9 $T71_2N$	HA of A/Shanghai/1/2013 $T71_2N$	Jan Baumann
pCAGGS-HA-H7N9 $N116_2D$	HA of A/Shanghai/1/2013 $N116_2D$	Jan Baumann
pCAGGS-HA-H7N9 $T71_2N$, $N116_2D$	HA of A/Shanghai/1/2013 $T71_2N$ $N116_2D$	Jan Baumann
pCAGGS-HA-Marseille	HA of A/swine/Marseille/2260/1980	Jan Baumann
pGL4.73 [hRluc/SV40]	encodes for *Renill*a luciferase reporter gene controlled by an SV40 promoter	Provided by Prof. Dr Friedemann Weber
pTM-Luc	*Firefly* luciferase gene	Promega, Mannheim; Germany

HA accession numbers: [a], KT715448; [b], EPI350266; [c], KT715456; [d], AF117241; [e], CY125894; [f], EPI8443; [g], NC_026425.

2.11 Viruses

H1N1 Viruses

Virus	Subtype	HA Accession No.
Avian		
A/duck/Alberta/35/1976[1]	H1N1	KT715447[a]
A/duck/Bavaria/1/1977[2]	H1N1	KT715448[a]
A/duck/Bavaria/2/1977[2]	H1N1	KT715449[a]
A/duck/Schleswig/21/1979[2]	H1N1	KT715450[a]
A/coot/Schleswig/4/1979[2]	H1N1	KT715446[a]
A/coot/Schleswig/2/1980[2]	H1N1	KT715445[a]
Avian-like swine		
A/swine/Arnsberg/6554/1979[2]	H1N1	KT715451[a]
A/swine/OLI/1/1980[2]	H1N1	KT715452[a]
A/swine/Marseille/2260/1980[2]	H1N1	KT715456[a]
A/swine/Italy/v147/1981[2]	H1N1	KT715455[a]
A/swine/Germany/2/1981[2]	H1N1	KT715453[a]
A/swine/Germany/S27/1981[2]	H1N1	KT715454[a]
A/swine/Italy/215990-3/2005[3]	H1N1	GQ175964[b]
A/swine/England/453/2006[4]	H1N1	CY116206[b]
A/swine/Italy/50175/2007[3]	H1N1	FJ770258[b]
Swine viruses with human-like HA		
A/swine/Italy/30019-2/2007[3]	H1N2	FJ770266[b]
A/swine/Italy/50127/2007[3]	H3N2	EPI162378[b]
Classical swine		
A/Thailand/271/2005[5]	H1N1	EF101749[b]
A/Illinois/09/2007[6]	H1N1	EPI482788[b]
A/Iowa/02/2009[6]	H1N1	EPI482799[b]
A/South Dakota/03/2008[6]	H1N1	JF758482[b]
Human		
A/HK/1/1968[7]	H3N2	EPI8443[b]
A/Hamburg/05/2009[1]	H1N1	EPI350266[b]

[a], *viruses sequenced in this work.*
[b], *sequence obtained from GISAID EpiFlu™ Database (www.platform.gisad.org).*

[1], repository of the Institute of Virology, Philipps University, Marburg, Germany.
[2], Christoph Scholtissek at the Institute of Medical Virology, Justus Liebig University, Giessen, Germany.
[3], repository of the Istituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia Romagna, Parma, Italy.
[4], Sharon Brookes and Ian Brown, Animal and Plant Health Agency, Addlestone, Surrey, United Kingdom.
[5], Ian Barr, WHO Collaborating Centre for Influenza, Melbourne, Victoria, Australia.
[6], Alexander Klimov and Amanda Balish, Centers for Disease Control and Prevention, Atlanta, GA.
[7], H3N2, Earl Brown, University of Ottawa, Ottawa, Canada.

H7Nx Viruses

Virus	Subtype	HA Accession No.[a]
H7 European wild bird		
A/duck/HK/293/1978[1]	H7N2	AAC54379
A/mallard/Netherlands/12/2000[2]	H7N3	ABI84599
A/mallard/Italy/33/2001[2]	H7N3	AAT37406
A/mallard/Sweden/56/2002[2]	H7N7	AAY46207
A/mallard/Sweden/102/2002[2]	H7N7	AAY46216
A/mallard/Sweden/105/2002[2]	H7N7	AAY46219
A/mallard/Sweden/106/2002[2]	H7N7	AAY46220
A/mallard/Sweden/64/2003[2]	H7N7	AEL99955
A/mallard/Netherlands/9/2005[2]	H7N7	ADQ20635
H7 North American wild bird		
A/mallard/Alberta/279/1977[1]	H7N3	ABB87784
A/green winged teal/Alberta/228/1985[1]	H7N3	ABB87800
A/ruddy turnstone/DE/2378/1988[1]	H7N7	ABB87822
A/laughing gull/DE/22/2002[1]	H7N3	AEO94612
H7 European poultry		
A/turkey/Italy/977/1999[3]	H7N1	ABS89321
A/turkey/Italy/2732/1999[3]	H7N1	ABV01277
A/turkey/Italy/3560/1999[3]	H7N1	AAK58937
A/turkey/Italy/8912/2002[3]	H7N3	ABO44178
A/turkey/Italy/251/2003[3]	H7N3	ABO44156
H7 North American poultry		
A/turkey/MN/1200/1980[1]	H7N3	ABI84683
A/turkey/MN/1/1988[1]	H7N9	ABI84694

[a], sequence obtained from GISAID EpiFlu™ Database (www.platform.gisad.org).

[1], viruses kindly provided by Robert Webster, St. Jude Children's Research Hospital Memphis, Tennessee, USA.
[2], viruses kindly provided by Ron AM Fouchier, Department of Viroscience, Erasmus MC, Rotterdam, Netherlands.
[3], viruses kindly provided by Research and Innovation Department, Istituto Zooprofilattico Sperimentale delle Venezie, OIE/FAO and National Reference Laboratory for Newcastle Disease and Avian Influenza, OIE collaborating Center for Diseases at the Human-Animal Interface, Padova, Italy.

Viruses of other subtypes

Virus	Subtype	HA Accession No.[a]
A/mallard/Alberta/119/1998[1]	H1N1	AGG27509
A/mallard/Alberta/205/1998[1]	H2N9	AY633196
A/mallard/Alberta/290/1998[1]	H3N8	AY633252
A/mallard/Alberta/47/1998[1]	H4N1	CY004925
A/duck/Minnesota/1525/1981[1]	H5N1	CY014726
A/gull/Netherlands/04/2007[2]	H13N6	Not available
A/mallard/Guryev/263/1982[1]	H14N5	Not available
A/gull/Netherlands/01/2007[2]	H16N3	Not available

[a], sequence obtained from GISAID EpiFlu™ Database (www.platform.gisad.org)
[1], viruses kindly provided by Robert Webster, St. Jude Children's Research Hospital Memphis, Tennessee, USA.
[2], viruses kindly provided by Research and Innovation Department, Istituto Zooprofilattico Sperimentale delle Venezie, OIE/FAO and National Reference Laboratory for Newcastle Disease and Avian Influenza, OIE collaborating Center for Diseases at the Human-Animal Interface, Padova, Italy.

Recombinant H3N2 viruses

Virus	Subtype	Description
rHK	H3N2	Recombinant A/Hong Kong/1/1968
rHK/R5 [a]	H3N2	I60R, N81D, K92N, G144A and S193N in rHK68 HA
rHK/R5+1 [a]	H3N2	D60G mutant in R5 HA

[a], for detailed description see Van Poucke et al., 2015.

3 Methods

3.1 Molecular methods

3.1.1 Preparation and transformation of competent *Escherichia coli*

TSS buffer is used to create transformation-competent XL1-blue Escherichia coli bacteria, which are able to take up circular plasmid DNA after heat shock treatment. The selection of successfully transformed bacteria is performed using a plasmid-coded antibiotic resistance gene. Only bacteria which have incorporated a transformed plasmid, are able to grow on growth medium containing selective antibiotics.
20 µl of XL1-blue *E. coli* bacteria were incubated in 5 ml LB medium at 37°C with shaking over night. From this overnight culture, 200 µl bacteria were transferred into fresh LB medium without antibiotics and incubated for two to four hours at 37°C on a shaker until an OD_{600nm} of 0.5 to 0.8 was reached. Then the culture was directly transferred onto ice and stored for 30 min, followed by low speed centrifugation (3000 rpm) for 10 min. The supernatant was discarded, and the pellet was re-suspended in 2 ml TSS buffer. 200 µl of the bacteria were mixed with 50 to 100 ng plasmid DNA and incubated on ice for another 30 min. After heat shock treatment at 42°C for 45 sec the complete bacteria solution was plated on agarose plates containing ampicillin. Plates were incubated over night at 37°C to allow bacteria growth. Single colonies were used for further plasmid expansion.

3.1.2 Plasmid DNA preparation from *Escherichia coli*

The plasmid isolation from *E. coli* was done with commercial kits. Depending on the isolation scale, either the peqGOLD Plasmid Miniprep Kit I (PeqLab) for small or the HiSpeed Plasmid Maxi Kit (Qiagen) for large volumes was used. Both systems are based on alkaline cell lysis. Plasmid containing *E. coli* colonies were grown in 5 ml ampicillin-supplemented LB medium over night at 37°C. The overnight culture was either used directly for plasmid isolation or for bacteria amplification. For bacteria growth 200 ml ampicillin-containing LB medium were inoculated with 500 µl bacteria

suspension and incubated over night at 37°C. After growth, cells were pelleted (4000 rpm, 15 min), and plasmid DNA was isolated according to manufacturers' instructions.

3.1.3 Spectral-photometric quantification of DNA

The absorption maximum of DNA is 260 nm and can be used for DNA quantification in solution. The quantification is based on the Lambert Beer equation:

$$A = \varepsilon bc$$

A, *absorption*
ε, *molar coefficient*
b, path length
c, concentration

The molar coefficient for double stranded DNA at 260 nm is 0.020 $(\mu g/ml)^{-1}$ cm^{-1} and the cuvette path length is 10 mm. Consequently, the concentration of DNA is calculated using the formula:

$$DNA\ concentration\ \left[\frac{\mu g}{ml}\right] = A_{260} \cdot 50 \frac{\mu g}{ml}$$

A, absorption

For quantification of the isolated DNA, the concentration of 1 µl eluted DNA was determined using a NanoDrop photometer.

3.1.4 Restriction enzyme digestion of double stranded DNA

Using restriction endonucleases, double stranded DNA can be cut at specific nucleotide sequence motifs. Depending on the restriction enzyme, specific 5' and 3' ends are created which can be used for subsequent ligation of DNA fragments. The restriction enzymes used in this study are listed in **2.7** including their specific recognition sites.

All restriction reactions were performed according to the manufacturers' instructions using recommended buffers, temperatures and incubation times.

3.1.5 DNA dephosphorylation

To avoid religation of endonuclease digested plasmids, DNA 5' ends were dephosphorylated using Shrimp Alkaline Phosphatase (SAP). Directly after endonuclease treatment, 3 units of SAP per 1 µg DNA were added to the reaction followed by incubation at 37°C for 2 h. The enzyme activity then was heat inactivated at 65°C for 15 min.

3.1.6 DNA ligation

Restricted DNA fragments were ligated into dephosphorylated, linearized vector DNA used for transient expression in cells. To facilitate covalent binding of free 3'OH groups with free 5' phosphate residues, a T4-DNA ligase was used. The linearized vector DNA was mixed with restricted insert DNA at a ratio of 1:3 or 1:6. One unit of T4-DNA ligase was added per 10 µl ligation mixture followed by overnight incubation at 16°C.

3.1.7 DNA amplification by polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) allows amplification of specific DNA fragments from a complex DNA mixture using sequence-specific oligonucleotide primers. The reaction can be divided into three parts which are repeated in a certain number of cycles. In the first step, double stranded DNA is denatured into single strands by high temperature. The subsequent cooling allows the short oligonucleotide primers to anneal at specific nucleotide sequences flanking the DNA sequence of choice. Following this hybridization, a DNA-dependent DNA-polymerase synthesizes the complement DNA strand starting from the primer's 5' ends, this step is called elongation. Multi-cycle repetition of these steps leads to an exponential enrichment of specific DNA fragments. To increase amplification accuracy, all PCR reactions were done using a *Pfu* polymerase, isolated from the archaea *Pyrococcus furiosus*. This polymerase is able to

proof read synthesized DNA by a 3'-5' exonuclease activity, which decreases the mutation rate to 10^{-6} nucleotide exchanges per site. The elongation time for each DNA fragment was calculated based on the *Pfu* polymerase synthesis capacity of 2,000 bases per minute,. The temperature of primer annealing is estimated according to the primer melting points, which were calculated using the Wallace-Ikatura equation:

$$T_m[°C] = 2(L + G + C)$$

L, *primer length*
G, *number of guanine residues*
C, *number of cytosine residues*

Reaction mix (50 µl):

dH_2O	37 µl
Pfu-reaction buffer	5 µl
dNTP-mix [10 mM]	2 µl
forward primer [20 pmol]	2 µl
reverse primer [20 pmol]	2 µl
DNA template [50-100 ng/µl]	1 µl
Pfu DNA polymerase	1 µl

PCR program:

Initial denaturation	95 °C	5 min	
Denaturation	95 °C	30 sec	30 cycles
Annealing	50 - 62 °C	30 sec	
Elongation	72 °C	2 min/kb	
Final Elongation	72°C	10 min	
	4 °C	∞	

All PCR reactions were performed in a PCR Thermo Cycler 2720.

3.1.8 One-step RT-PCR

For direct amplification of DNA from isolated viral RNA a one-step RT-PCR was performed using the OneStep RT-PCR Kit (Qiagen). The kit includes all necessary enzymes including the reverse transcriptases Omniscript™ and Sensiscript™ as well as a HotStar Taq-Polymerase™. The PCR was performed according to manufacturers' instructions.

3.1.9 Site directed mutagenesis

Site directed mutagenesis allows for the introduction of single or multiple nucleotide exchanges as well as insertions and deletions into a given plasmid. In this work a PCR mutagenesis protocol using two complementary primers covering the target sequence was applied. Both primers contain single or double nucleotide exchanges to introduce mutations. Using both primers the plasmid was amplified by PCR. As a consequence, this *in vitro* system synthesizes mutated plasmid DNA as linear fragments. In contrast to the parental plasmid, the linear DNA is unmethylated. This allows the specific degradation of the not mutated parental DNA by *Dpn*I digestion, cutting methylated target motifs (5'…$G^{m6}ATC$…3') only. Subsequently, the linear mutated plasmid was transformed into *E. coli* XL1-blue bacteria, which facilitate circularization and amplification. PCR based site directed mutagenesis was performed as followed:

Reaction mix (50 µl):

dH_2O	37 µl
Pfu-reaction buffer	5 µl
dNTP-mix [10 mM]	2 µl
forward primer [20 pmol]	2 µl
reverse primer [20 pmol]	2 µl
DNA template [50 ng/µl]	1 µl
Pfu DNA polymerase [2.5 U/µl]	1 µl

PCR program:

Initial denaturation	95 °C	5 min	
Denaturation	95 °C	30 sec	20 cycles
Annealing	50 - 65 °C	30 sec	
Elongation	68 °C	2 min/kb	
Final Elongation	68°C	10 min	
	4 °C	∞	

*Dpn*I digestion

PCR reaction	50 µl
Tango™ Buffer (10x)	5 µl
*Dpn*I enzyme	10 U

The *Dpn*I digestion was performed for one hour at 37°C followed by enzyme inactivation at 80°C for 20 min. Subsequently, the mutagenesis reaction was transformed into transformation competent *E. coli* XL1-blue bacteria. Selection for successful transformation was carried out via growth on ampicillin-containing LB-agar culture plates. Single bacteria colonies from these plates were then used for further plasmid amplification and plasmid isolation.

3.1.10 Electrophoretic separation of DNA fragments

Gel electrophoresis allows separation of DNA fragments according their size. This method is based on the movement of the negatively charged DNA in an electrical field from the negative electrode (cathode) to the positive electrode (anode). The movement speed depends on the fragment size, the bigger the slower, and the pore diameter, the smaller the slower. The pore diameter is defined by the agarose concentration used for agarose gel preparation.

Samples were mixed with 5x DNA sample buffer and transferred to a 0.8 % agarose gel in TBE running buffer. DNA-marker GeneRuler™ 1kb ladder and O´Gene Ruler™ 100 bp DNA ladder were used as size standard. The electrophoresis was performed at

120 V and 50 mA. The gel was stained using ethidium bromide which intercalates into double stranded DNA structures. The DNA was visualized using UV-light and the agarose gel was analysed using a GelDoc 2000 system.

3.1.11 Purification of PCR products

To purify DNA sample from enzymes, nucleotides, and primers, a QIquick® PCR purification Kit (Qiagen) was used. The protocol is based on the ability of DNA to bind silica membranes under salty high pH conditions (> pH 7.5). After the binding step, contaminations were washed away using ethanol-containing buffers. The purification was done according to manufacturers' instructions and purified DNA was eluted in 50 µl deionized water.

3.1.12 DNA sequencing

In order to sequence viral gene segments, viral RNA was isolated using the QIAamp® viral RNA Mini Kit. The isolation was performed according to manufacturers' instructions. Briefly, 140 µl virus stock was inactivated using a highly denaturing buffer (AVL buffer). RNA was precipitated with 560 µl ethanol (96%) and RNA was bound to a silica membrane (QIAmp Mini column). After washing the pure RNA was eluted in 50 µl RNAse-free water. The extracted viral RNA was transcribed into cDNA and further amplified (section **3.1.8**) using universal HA specific primers (Hoffmann et al., 2001). Following gel purification (section **3.1.11**), 100 to 200 ng of purified viral cDNA was sent for Sanger sequencing (Sanger et al., 1977) to the SeqLab company (Göttingen; Germany). Sequence analysis was done using Geneious 5.5.9 (Drummond et al., 2011; Available from www.geneious.com).

3.2 Cellular methods

3.2.1 Cell culture

Madin-Darby canine kidney (MDCK), human embryonic kidney (HEK 293T), and Henrietta Lacks cervical cancer (HeLa) cells, as well as baby hamster kidney fibroblasts (BHK) were maintained in Dulbecco´s modified Eagle´s medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin (100 IU ml^{-1}/100 µg ml^{-1}) and 2 mM glutamine (growth medium). Cells were grown in cell culture flasks and passaged when the cells reached complete confluency. For cell passaging DMEM medium was removed and cells were washed twice with PBS^{++}. To detach cells from plastic, trypsin-EDTA was added (0.5 ml for 25 cm^2 flask; 2 ml for 75 cm^2 flask). After detachment cells were re-suspended in the fresh growth medium (4.5 ml for 25 cm^2 flask; 8 ml for 75 cm^2). A proportion of the cells was transferred to a new growth medium containing flask. Cell lines were maintained at 37°C, 5% CO_2 and 95% humidity.

3.2.2 Transfection of eukaryotic cells

Transfection is used to introduce plain linear or circular DNA into eukaryotic cells, in order to specifically control gene expression or to synthesize foreign genes. For this purpose, a cationic liposomal agent is used (Lipofectamine™ 2000; Invitrogen). Mixing Lipofectamine with DNA results in the formation of liposome/DNA complexes, which are endocytosed by target cells. Lipid-mixing then leads to release of the DNA into the cytoplasm. Subsequently, the DNA enters the cell nucleus where transcription occurs.

Plasmid DNA (0.8 – 1 µg) in OptiMEM medium was mixed with OptiMEM containing Lipofectamine (amount depends on plasmid concentration; described in manufacturers' instructions) at a ratio of 1:1, followed by incubation at room temperature for 20 min to allow DNA uptake into liposomes. HEK393 or HeLa cells grown to 70 – 80 % confluence were washed twice with PBS^{++}, the transfection mix was added and cells were incubated for 4 h at 37°C, 5% CO_2 and 95% humidity. Transfection mixture then

was discarded, cells were covered with growth medium and incubated for 24 to 48 h at 37°C, 5% CO_2 and 95% humidity.

3.3 Virological methods

3.3.1 Virus growth

Avian, human, and porcine viruses were grown either in MDCK cells or in embryonated chicken eggs. Viruses used in this study are MDCK-grown if not indicated otherwise (section **2.11**). After virus growth stocks were prepared for all viruses and stored at -80°C. The HA of all viruses was sequenced and virus titres were determined using hemagglutination assay and focus-forming assay. An additional plaque titration was performed for viruses used in growth kinetics.

3.3.1.1 Virus growth in cell culture

MDCK cells grown to 80 – 90 % confluency were washed twice with PBS^{++} followed by virus infection with an MOI (multiplicity of infection) of either 0.001 or 0.005 depending on viral replication efficiency. MOI is defined as virus particle per cell and is calculated as follows:

$$MOI = \frac{cell\ number}{virus\ titre\ [PFU/ml]} \cdot target\ MOI$$

MOI, multiplicity of infection
PFU, plaque forming units

For virus infection, infection medium (DMEM; 0.1% BSA; 1% Penicillin/Streptomycin; 1% L-Glutamine) supplemented with 1 µg/ml TPCK-treated trypsin was mixed with the desired amount of virus stock. After infection, cells were incubated at 37°C, 5% CO_2 and 95% humidity for 72 to 96 h depending on virus growth. The virus-containing supernatant from infected cultures was harvested, clarified by low-speed centrifugation (4000 rpm; 10 min) and used without further purification.

Virus stocks were stored at -80°C and titrated using the focus forming assay (section **3.3.2**).
For studies of receptor-binding properties, after clarification, viruses were concentrated by ultracentrifugation (25,000 rpm; 1.5 h). The virus pellet was re-suspended in 300 µl glycerol (50% in dH_2O) each and stored at -20°C.

3.3.1.2 Virus growth in embryonated eggs

For virus growth 11-day-old eggs were screened for living chicken embryos. Eggs containing living embryos were disinfected with iodine, and 100 µl infection medium containing 500 – 2,000 focus forming units (FFU) per ml of virus, was injected directly into the allantoic fluid (injection needle: 0.55 x 25 mm). The puncture was closed using Ponal-Glue (Henkel). Infected eggs were incubated for 48 h at 37°C and 95% humidity. After incubation eggs were stored over night at 4°C to allow blood vessel contraction. The virus-containing allantoic fluid was removed using a plastic pipette and clarified by low speed centrifugation (4000 rpm; 10 min). Aliquoted virus stocks were stored at -80°C.

3.3.2 Focus forming assay

Using the focus forming assay (Matrosovich et al., 2007), the amount of infectious particles can be determined as focus forming units (FFU). By omitting trypsin, the viral HA is not cleaved into the fusion competent form, which prevents further virus spread to neighbouring cells. The resulting individual infected cells can be stained immunohistochemically (section **3.3.3**), thereby every infected cell represents a single infectious viral particle or focus forming unit. Infected cells were counted, and the virus titre was calculated according to the virus dilution as FFU/0.1 ml.
For titration, confluent monolayers of MDCK cells grown in 96-well plates were inoculated with 0.1 ml of serial 10-fold virus dilutions in infection medium. The titration was performed in three replicates per dilution. Cells were incubated at 37°C, 5% CO_2 and 95% humidity. Eight hours post infection cells were fixed with 4%

paraformaldehyde and stained immunohistochemically for influenza NP expression as described below (section **3.3.3**).

3.3.3 Plaque assay

In order to detect infectious viral particles, which are able to initiate multi cycle replication, viruses are titrated using the avicel plaque test. The viscous avicel overlay allows only short distant diffusion of released virions. As a consequence, virus spread results in local plaque formation with each detected plaque representing one infectious particle. The resulting titer is indicated as plaque forming unit per milliliter (PFU/ml). The assay was performed as described before (Böttcher et al., 2006a). Briefly, titration was carried out using 90% confluent MDCK cells in 6-well plates. The cells were washed twice with PBS^{def} followed by infection with 10-fold dilutions of the virus sample in 1 ml infection medium. After 1h of virus adsorption at 37°C, 5% CO_2 and 95% humidity 2 ml avicel overlay containing TPCK-trypsin (1µg/ml) was added to each well and cells were incubated at 37°C, 5% CO_2 and 95% humidity. Two days post infection the overlay medium was removed and cells were washed twice with PBS^{def} to remove residual medium. The cells were then fixed with 4% paraformaldehyde for 1 h at 4°C and stained immunohistochemically for Influenza A virus NP.

3.3.4 Immunohistochemical staining of virus-infected cells

Influenza virus infected cells were stained as described before (Matrosovich et al., 2007). Paraformaldehyde-fixed cells were permeabilized with 0.3% Triton X-100. To detect viral infection, cells were incubated with a monoclonal mouse anti-influenza-NP antibody (in 10% horse serum; 0.05% Tween 80 in PBS^{def}; kindly provided by Alexander Klimov, Center for Disease Control; USA) for two hours. Cells were washed three times with PBS^{def} followed by treatment with peroxidase-labelled polyclonal rabbit anti-mouse serum (Dako; 10% horse serum, 0.05% Tween 80 in PBS^{def}). After washing with PBS^{def} 50 µl TrueBlue™ peroxidase substrate (KPL; supplemented with 0.1% H_2O_2;) was added to stain infected cells. The reaction was stopped by washing

with deionized water. Infected cells were counted for virus dilutions that produced between 50 and 200 infected cells and recalculated to focus forming units per 0.1 ml.

3.3.5 Inhibition of infection by the lysosomotropic agent ammonium chloride

Inhibition of virus infection by lysosomotropic agents is based on their ability to accumulate in acidic cell compartments and prevent their acidification by proton absorption. Ammonium chloride (NH_4Cl) is known to accumulate in endosomal vesicles and interfere with their acidification during lysosome maturation. In the course of ATPase-driven protonation of the endosome, NH_4Cl inhibits a variety of lysosomal hydrolases (de Duve et al., 1974; Ohkuma and Poole, 1978).

NH_4Cl elevates the endosomal pH and prevents the HA conformational transition and subsequent membrane fusion in a dose-dependent manner. Interpretation of the results can be made as follows: The less NH_4Cl needed for infection inhibition, the lower the pH necessary for fusion induction (Matlin et al., 1981).

In order to quantify the inhibitory effect of NH_4Cl, monolayers of confluent MDCK cells were infected with 200 FFU influenza virus in 0.1 ml infection medium in the presence of different concentrations of ammonium chloride (from 0 mM to 2.5 mM) in a 96-well plate format (Baumann et al., 2015; Krenn et al., 2011). The infection was performed without trypsin to limit replication to one cycle. The cells were incubated for 16 hours followed by immunohistochemical staining for viral NP (section **3.3.3**).

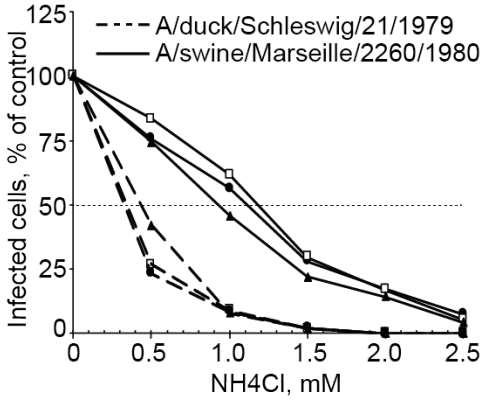

Figure 3.1: Example of inhibition of viral infection by NH_4Cl. *Results of three replicate experiments performed on the same day are shown for A/duck/Schleswig/21/1979 (solid lines) and A/swine/Marseille/2260/1980 (dashed lines). Concentrations of NH_4Cl that caused 50% infection inhibition were determined for each replicate by interpolation.*

The number of infected cells was quantified and infection efficiency was calculated as percentage in respect to infection without NH_4Cl. Dose-response curves were plotted (**figure 3.1**) and 50% infection inhibition (IC_{50}) by NH_4Cl was determined by linear interpolation for each replicate. All experiments were performed in triplicates and repeated independently at least twice. Results of all individual replicates were averaged.

3.3.6 Virus inactivation at low pH

The energy barrier to induce the HA conformational transition can also be overcome by low pH and denaturing urea in *vitro*. The structural rearrangement into the fusion competent HA abolishes receptor-binding capacity and results in virus inactivation. The lower the urea concentration or the higher the pH needed to induce this transition, the less stable the virus. In this work virus inactivation at acidic pH was used to determine viral stability (Baumann et al., 2015).

To determine the pH of virus inactivation, viruses were adjusted to 10,000 - 20,000 FFU followed by exposure to buffers with pH ranging from pH 7.0 to 5.0 in MES-buffer (100 mM MES, 150 mM NaCl, 0.9 mM $CaCl_2$, 0.5 mM MgCl in dH_2O; pH adjusted with NaOH) for 15 min. The mixtures were diluted 100-fold with infection medium for neutralization. Confluent monolayers of MDCK cells grown in 96 well-plates were infected with 0.1 ml neutralized virus solution per well. Eight hours post infection cells were immunohistochemically stained for influenza NP expression (section **3.3.3**). Infected cells were counted and plotted in inhibition curves (**figure 3.2**). Using linear interpolation, the pH of 50% infection inhibition (pH_{inact}) was determined. Experiments were done in triplicates. Presented values of pH_{inact} represent mean values from at least two independent experiments.

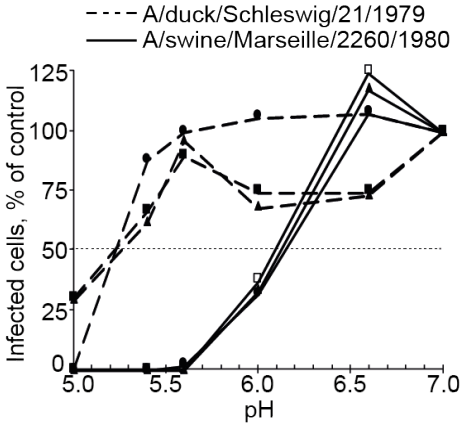

***Figure 3.2: Example of virus inactivation at low pH**. Results of three replicate experiments performed on the same day are shown for A/duck/Schleswig/21/1979 (solid lines) and A/swine/Marseille/2260/1980 (dashed lines). pH values that caused 50% infection inhibition were determined for each replicate by interpolation.*

3.3.7 Growth kinetics in HTBE cultures

To mimic virus replication in the human respiratory tract 6-week-old differentiated human tracheobronchial epithelial (HTBE) cultures (kindly provided by Tatyana Matrosovich) were infected with 10^4 plaque forming units (PFU) of virus. In order to do that, cells were washed five times with PBS^{def} followed by infection with 200 µl DMEM containing 10^4 PFU of virus. After incubation for 1 h at 33°C the inoculum was removed and cells were washed once with 300 µl DMEM. For virus replication cells were incubated over the course of six days at 33°C, 5% CO_2 and 95% humidity.
Virus sampling was done regularly at a 24 h basis. Released viruses were harvested by washing the apical side of the cells with 0.4 ml DMEM for 30 min at 33°C. The collected material was titrated (section **3.3.2**). The experiment was performed in four to five replicates.

3.3.8 Hemagglutination assay

The influenza A surface protein HA binds sialo-glycans on the surface of human or chicken erythrocytes. This results in the formation of a cross-connected erythrocyte-virus network that prevents red blood cells from sedimentation. If the virus concentration is reduced, a threshold will be reached where remaining virus particles

are not sufficient to cross connect red blood cells to prevent sedimentation. The dilution at which this threshold is reached allows a semi-quantitative prediction about the virus concentration. As the virus solution may contain HA-exhibiting incomplete virions, no differentiation between infectious and non-infectious particles can be made. In order to determine the HA titre, virus solutions were diluted in 2-fold steps in a 96-well microtiter plate. For each dilution, 50 µl of the previous dilution (with the first dilution being the original virus solution) was mixed with 50 µl PBS^{def}. 50 µl of a chicken red blood cell solution (1%) was added to each well followed by incubation for 1 to 3 h at 4°C. Afterwards, the dilution at which red blood cells sedimented was determined, and the hemagglutination titre (HA units; HAU) was calculated according to the formula,

$$HAU = \frac{1}{Virus\ dilution\ of\ sedimentation}$$

.

3.3.9 Quantification of viral hemolytic activity

Based on the HA-mediated agglutination of erythrocytes, this assay allows measurement of the pH of HA-mediated hemolysis. Viruses are agglutinated to human red blood cells followed by a stepwise decrease of the pH. At a threshold pH HA conformational transition into its fusion competent structure is initiated. This results in HA-mediated fusion induction leading to erythrocyte lysis which can be measured by detection of peroxidase activity of released hemaproteins (Montaño and Morrison, 1999). As hemolysis induction exclusively depends on HA fusion activity, this assay allows the exclusive investigation of HA pH dependency and influences of other pH dependent viral proteins.

HA-mediated hemolytic activity was measured as described by Baumann et al., 2015. 64 HAU of virus were incubated with a 0.03 % solution of human erythrocytes (hRBC, human red blood cells) for 1 h on ice. After agglutination mixtures were dispensed a 50 µl in a 96-well plate. The mixtures were incubated in different pH conditions ranging from pH 7.0 to 5.0 in sodium acetate buffer (0.1 M sodium acetate buffer adjusted to pH 5.0 – 7.0 with NaOH) for 30 min at 37°C followed by neutralization with Tris/HCl, pH 7.3. For comparison complete hemolysis was initiated using triton X-100 (0.01%). Non-lysed erythrocytes and cell debris were removed by centrifugation (10 min, 2000

rpm) and 50 µl of the supernatant was used for detection of hemolytic activity using 3,3′,5,5′-Tetramethylbenzidine (TMB; 0.05 M sodium acetate buffer, pH 5.2; 1:100 TMB-stock; 0.03% H_2O_2; Martin et al. 1984). Peroxidases released from lysed red blood cells oxidise TMB (**figure 3.3**) and the resulting product can be detected photometrically.

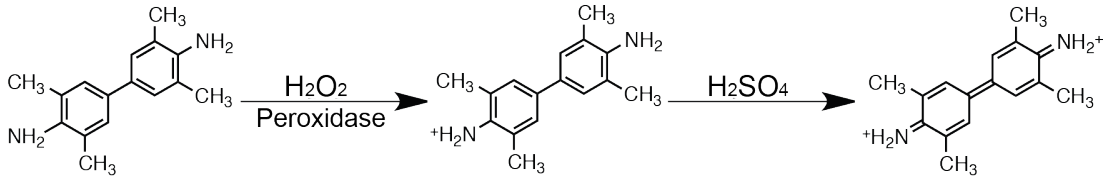

Figure 3.3: Conversion of TMB *into partially and fully protonated forms by peroxidase activity of hemaproteins in the presence of H_2O_2 and H_2SO_4.*

After incubation for 30 min at room temperature the reaction was stopped with 25 µl 5% sulphuric acid and absorption at 450 nm was measured using a microplate reader.

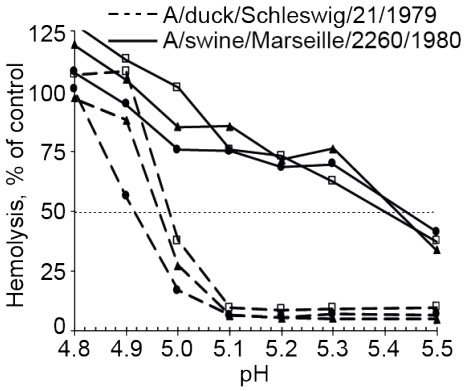

Figure 3.4: Example of pH dependency of virus-mediated hemolysis*. Results of three replicate experiments performed on different days are shown for A/duck/Schleswig/21/1979 (solid lines) and A/swine/Marseille/2260/1980 (dashed lines). pH values that induced 50% hemolytic activity were determined for each replicate by interpolation.*

The pH of 50% hemolytic activity (pH_{hem}) was determined for each virus by interpolation of hemolysis/pH curves (**figure 3.4**).

3.3.10 Flow cytometric analysis

Using flow cytometry cells can be sorted according their cell shape and structure. In combination with fluorescence-labeled antibodies the cell proportion expressing specific proteins can be determined. In this study, this method is used to determine HA surface expression.

80% confluent HeLa cells grown in 4 cm^2 multiwell plates were transfected with 1 µg of HA-encoding or empty pCAGGS plasmids (section **3.2.2**). The cells were incubated at 37°C, 5% CO_2 and 95% humidity. After 24 h cells were scratched from the plates. In order to cleave HA 1 µg/µl TPCK-trypsin was added followed by 30 min incubation at 37°C. Cells were pelleted (1200 rpm; 10 min) and re-suspended in 200 µl ELISA buffer containing anti-H1 HA serum (1:200). After 1 h shaking at room temperature cells were washed twice with washing buffer (0.05% Tween in PBS^{def}) and re-suspended in 200 µl ELISA buffer containing FITC (Fluorescein isothiocanate)-labelled anti-rabbit antibodies (1:100). Cells were incubated for 30 min at room temperature, washed twice with washing buffer and re-suspended in 500 µl PBS^{def}. 10,000 cells were counted using a cell sorter (BD Biosciences-US). Cells were excited at 495 nm and fluorescence signals were detected at 520 nm. FITC fluorescence of cells transfected with empty vector was subtracted from sample fluorescence and percentage of FITC positive cells was calculated.

3.3.11 Quantification of syncytia formation by light microscopy

Plasmid driven HA expression in permanent cell lines such as HeLa and HEK293 cells leads to HA presentation at the cell surface. This can be used to study the pH of HA-mediated cell-to-cell fusion after incubation under different pH conditions. Fused cells which include multiple nuclei are known as syncytia and their formation can be observed microscopically.

The quantification of HA membrane fusion activity was performed as described previously (Reed et al., 2009). HeLa cell monolayers of 70 – 80% confluency grown in 6-well plates were transiently transfected with 1 µg HA-encoding pCAGGS-HA using Lipofectamine 2000 transfection reagent (section **3.2.2**). After incubation for 4 h at 37°C the transfection medium was replaced by infection medium. Sixteen hours post

transfection HA was cleaved by addition of 1 µg/ml TPCK-treated trypsin and incubation for 15 min at 37°C. Transfected cells were exposed to different low pH sodium acetate buffers ranging from pH 7 to pH 5.1 (145 mM NaCl; 20 mM sodium acetate) for 10 min. The supernatant was replaced by infection medium. After incubation for three hours at 37°C to allow fusion and syncytia formation, cells were fixed with methanol for 10 min and stained with Giemsa dye (Merck; 1:10 in dH_2O). Cells were washed with deionized water and dried. For quantitative analysis, pictures of five microscopic fields, chosen at random, were taken with a 300-fold magnification using a light microscope. For each image the percentage of nuclei within syncytia to total nuclei number was calculated.

3.3.12 Quantification of syncytia formation using luciferase reporter assay

Another approach to detect and quantify HA driven cell-to-cell fusion and syncytia formation uses measurements of activity of co-transfected *Firefly* luciferase. In this assay, a *Firefly* reporter gene regulated by a bacteriophage-derived T7-Polymerase-dependent promoter is used. On that account, *Firefly* luciferase is not synthesized in eukaryotic cells lacking a T7-Polymerase. The cells, transfected with HA and *Firefly* luciferase plasmids, were mixed with BHK cells constitutively expressing T7-polymerase. The subsequent low pH treatment leads to cell-to-cell fusion. The resulting syncytia formation is accompanied by the mixing of cytoplasmic components of the participating cells. As a consequence, the resulting syncytia contain both the T7-polymerase coding plasmid and an active T7-polymerase. The synthesized *Firefly* luciferase can then be used to quantify HA-mediated fusion activity.

In this work the luciferase-based quantification of syncytia formation was performed as described before by Su et al., 2008. HEK293 cell monolayers of 70 – 80% confluency grown in 6-well plates were transfected with 800 ng of pCAGGS-HA, 50 ng of a plasmid expressing *Renilla* luciferase and 400 ng of pTM1 plasmid coding for *Firefly* luciferase under the control of the T7 bacteriophage promoter (section **3.2.2**). Sixteen hours post transfection cells were scraped and mixed with equal amounts of BHK-T7 cells constitutively expressing T7-polymerase. After incubation at 37°C for 2 h HA expressed on the cell surface was cleaved adding 1 µg/ml TPCK-treated trypsin for

10 min at 37 °C. Cells were pelleted (1000 rpm; 5 min) and treated with sodium acetate buffers with pH from 5.0 to 7.0 for 3 min at 37°C. The supernatant was replaced by DMEM (supplemented with 0.1% BSA), and cells were incubated for 5 h (37°C, 5% CO_2 and 95% humidity) to allow cell-to-cell fusion and *Firefly* luciferase synthesis. Luciferase activity was measured using the Dual-Luciferase-Assay-Kit (Promega) according to the manufacturers' instructions. For quantitative analysis *Firefly* luciferase activity was normalized against the total amount of transfected cells showing *Renilla* luciferase activity. The pH of fusion induction was defined as pH at which *Firefly* luciferase activity was higher than the activity of mock transfected cells.

3.3.13 Quantitative analysis of viral receptor-binding

Quantitative and qualitative differences in viral receptor-binding properties were investigated using two assays, which are both based on HA binding to soluble receptor analogues. Viral binding was investigated either by direct binding to horse radish peroxidase (HRP)-labelled fetuin (Fet) or binding inhibition using synthetic sialyl glycopolymers (SGPs).

Virus receptor-binding specificity and avidity to α2,3- and α2,6-linked sialic acid receptors was determined using a solid phase assay described previously (Matrosovich and Gambaryan, 2012). The assay is based on binding of immobilized virus to soluble monospecific fetuin. To discriminate between human (α2,6) and avian (α2,3) type receptor-binding, HRP-linked asialo-fetuin was re-sialyated using α2,6- and α2,3-sialyltransferases, respectively. The resulting α2,6-Fet and α2,3-Fet only differ in the type of glycosidic linkage between the terminal sialic acid (Neu5Ac) residue and the penultimate galactose residue. To quantify receptor-binding to both fetuins peroxidase activity was determined.

Flat-bottomed 96-well plates were coated in batches of 25 plates. 510 ml of a 10 mg/ml fetuin working solution were prepared in PBS^{def}. 0.2 ml of the solution was added to each well, followed by overnight incubation at 4°C. Afterwards, the well contents were removed, the plates were washed three times with deionized water and air-dried at room temperature.

For each virus, 16 wells of a fetuin-coated plate were incubated with 0.05 ml virus solution per well for 1 h at 4°C. The supernatant was discarded, and non-specific

binding sites were blocked by adding 0.1 ml of 0.1% *Vibrio cholerae* sialidase-treated BSA (BSA-NA) in PBS^{def} per well for 1 h at 4°C. BSA-NA was prepared previously as follows. 50 ml of a 5 % BSA solution in PBS^{def} was prepared. One ml of Penicillin/Streptomycin (5000 U ml^{-1}/5000 µg ml^{-1}) was added and the pH was adjusted to 7.5. Then, one unit *Vibrio cholerae* sialidase was added and the solution was incubated for 24 h at 37°C. The sialidase was inactivated by incubation at 60°C for 24 h. The BSA-NA solution was aliquoted and stored at -20°C.

The following procedures were performed on ice. After removal of the blocking solution the plate was washed twice with 0.2 ml ice-cold washing buffer. An appropriate range of two-fold dilutions of α2,3-Fet and α2,6-Fet were prepared in reaction buffer. Two wells per virus were filled with 0.05 ml fetuin solution per dilution and fetuin type. After incubation for 1 h at 4°C the fetuin solution was discarded and the plate was washed five times on ice with washing buffer. To detect fetuin bound by viruses 0.1 ml/well substrate solution (0.01% TMB, 0.03% H_2O_2 in 0.05 M sodium acetate, pH 5.5) was added, followed by a 30 min incubation at room temperature. The reaction was stopped by adding 0.05 ml 5% H_2SO_4 per well, and absorbency was measured at 450 nm (A_{450nm}) using a microplate reader (Epoc, Biotek). Unspecific absorbency from wells containing no virus was subtracted from all sample values. The resulting data were converted to Scatchard plots (A_{450nm}/c as a function of A_{450nm}). Trend lines were drawn for α2,3-Fet and α2,6-Fet. Association constants (K_{ass}) were calculated using the formula:

$$K_{ass} = \frac{y_0}{A_{max}}$$

$K_{ass}, association\ constant$

$y_0, intercept\ of\ trendline\ with\ y\ axis$

$A_{max},\ intercept\ of\ trendline\ with\ x\ axis$

K_{ass} are expressed as micromolar amounts with higher values reflecting stronger binding.

3.4 Phylogenetic analysis

In order to determine the phylogenetic relationship of either H1 or H7 HAs, HA nucleotide sequences were obtained from the National Centre for Biotechnology Information (NCBI) Influenza Virus Resources Database (Bao et al., 2008; http://www.ncbi.nlm.nih.gov) and *GISAID EpiFlu™ Database (www.platform.gisaid.org/)*. HAs without published sequences were sequenced as described previously (section **3.1.12**) and submitted to the NCBI database (for accession numbers see **2.11**). All not redundant full-length sequences were aligned using the Muscle algorithm included in Geneious 5.5.9 (Drummond et al., 2011; Available from www.geneious.com). The conduction of the phylogenetic tree was done via the MEGA6 software (Tamura et al., 2013) using the minimal evolution method. The statistical likelihood of the H7 HA tree was determined using bootstrap analysis with 100 repetitions. H1 HA ancestral avian and swine amino acid sequences were predicted with the maximum-likelihood method using a Dayhoff matrix-based model included in MEGA6.

4 Results

4.1 Differences in the pH-dependent HA-mediated membrane fusion activity of avian and swine H1N1 viruses

In the late 1970s a European H1N1 duck virus transmitted to the European pig population and became endemic in Europe and Asia, forming the so-called Eurasian avian-like swine (EAsw) lineage (see section **1.6.2**). In order to study potential changes in the viral membrane fusion activity that emerged during avian-to-swine transmission, EAsw viruses isolated early after transmission to pigs (1979-1981) were compared with contemporary circulating H1N1 viruses from wild aquatic birds (**figure 4.1a**). The phylogenetic relationship between viruses used in this study is shown in **figure 4.1b**. In addition, a limited number of other swine and human viruses, including H1N2 and H3N2 swine viruses with human-virus-like HAs, viruses with "classical" swine HA isolated from humans after zoonotic infections, recent EAsw virus isolates, and two pandemic human viruses, were included in this study for comparison.

Only limited information about the passage history is available for the EAsw viruses and their closest avian ancestors. For this reason, the number of egg and cell culture passages is not known for some viruses used in this study. It cannot be excluded that viral growth in different cell culture systems alters the glycosylation status of viral glycoproteins or results in a changed composition of the cell derived viral envelope. In order to eliminate these potential influences on viral phenotypes, all viruses were re-grown in MDCK cells.

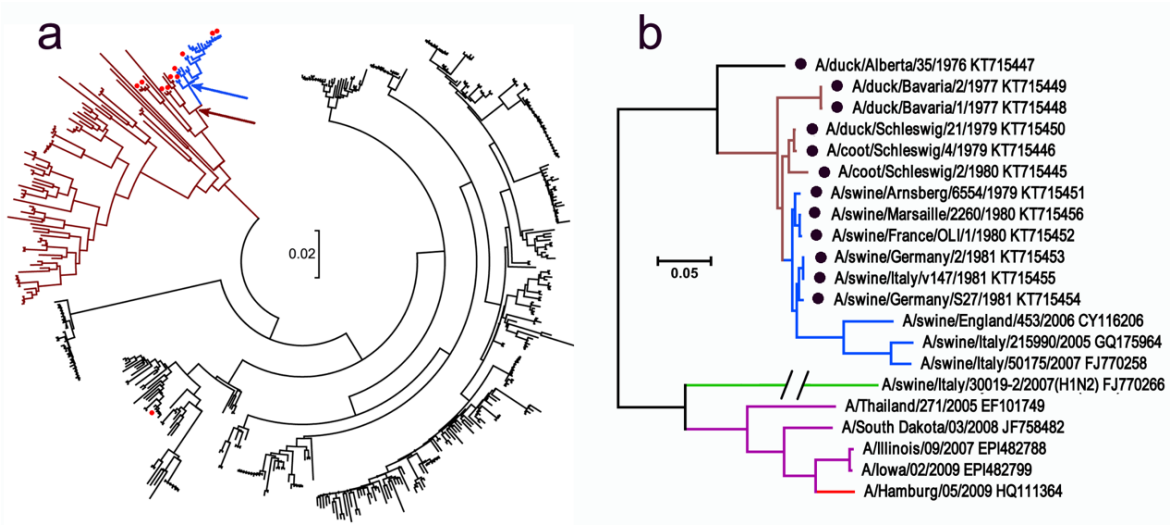

Figure 4.1: Phylogenetic relationship of H1 influenza virus HAs. a *The phylogenetic tree was built using 455 sequences of avian viruses from North America (black), Eurasia, Oceania and Africa (brown) and EAsw viruses (blue). Viruses used in this study are marked with red dots. The locations of the hypothetical first swine virus and its putative avian precursor are indicated by arrows (blue and brown, respectively).* ***b*** *Phylogenetic relationship of H1 viruses tested in this study. Colour code of genetic lineages: black, brown and blue, see panel (a); green, H1N2 swine viruses with human-like HA; purple, human isolates with classical swine HA; red, H1N1/2009 pandemic virus. Black dots indicate HA sequences determined in this study. HA sequences for A/Illinois/09/2007 and A/Iowa/02/2009 (Shu et al., 2012) were obtained from GISAID EpiFlu™ Database (www.platform.gisaid.org/). In both panels the scale bars represent units of nucleotide substitutions per site.*

The acidification of endosomes triggers the HA-mediated fusion of the viral and endosomal membrane and is essential for viral cell entry. In order to investigate variations of the pH of fusion between avian and EAsw viruses, infection of MDCK cells in the presence of the lysosomotropic agent ammonium chloride (NH_4Cl) was performed. NH_4Cl accumulates in acidic cellular compartments, such as endosomal vesicles, and interferes with their acidification in a dose dependent manner (Matlin et al., 1981). Thus, the NH_4Cl concentration correlates with the endosomal pH. NH_4Cl concentrations that inhibit infection by 50% (IC_{50}) were determined from dose-response curves. Data for individual viruses are summarized in **table 2,** mean values for avian, swine and human viruses are illustrated in **figure 4.2a**.

Table 2: Membrane fusion activity and stability of influenza viruses[a].

Virus[b]	Infection inhibition by NH_4Cl, IC_{50} (mM)			Hemolytic activity, $pH_{50\text{-}hem}$			Inactivation at acidic pH, $pH_{50\text{-}inact}$		
	Mean	± CI	*P*	Mean	± CI	*P*	Mean	± CI	*P*
Avian viruses									
A/duck/Alberta/35/1976	0.26	0.011	***	4.97	0.05		5.27	0.03	
A/duck/Bavaria/1/1977	0.50	0.007		5.20	0.06	***	5.31	0.11	
A/duck/Bavaria/2/1977	0.31	0.014	*	5.03	0.05		5.43	0.03	**
A/duck/Schleswig/21/1979	0.43	0.07		4.96	0.04		5.19	0.07	
A/coot/Schleswig/4/1979	0.55	0.07	*	5.05	0.03	**			
A/coot/Schleswig/2/1980	0.50	0.07		5.10	0.021	***			
Eurasian avian-like swine viruses									
A/swine/Arnsberg/6554/1979	0.99	0.16	***	5.07	0.07	*	5.75	0.017	***
A/swine/France/OLI/1980	1.04	0.21	***	5.29	0.10	***	5.64	0.03	***
A/swine/Marseille/2260/1980	1.13	0.13	***	5.36	0.05	***	5.91	0.11	***
A/swine/Italy/v147/1981	1.1	0.6	*	5.07	0.07	*			
A/swine/Germany/2/1981	0.95	0.16	***	5.21	0.09	**	5.75	0.20	***
A/swine/Germany/S27/1981	1.14	0.15	***	5.13	0.07	**	5.47	0.03	**
A/swine/Italy/215990-3/2005	1.43	0.05	***	5.39	0.024	***			
A/swine/England/453/2006	0.83	0.15	**						
A/swine/Italy/50175/2007	1.80	0.10	***	5.19	0.09	**			
Swine viruses with classical swine HA isolated from humans									
A/Thailand/271/2005	0.67	0.14	*	5.17	0.15	*			
A/Illinois/09/2007	0.75	0.20	*						
A/South Dakota/03/2008	0.65	0.10	*						
A/Iowa/02/2009	2.55	0.10	***						
Swine viruses with human-like HA									
A/swine/Italy/30019-2/2007 (H1N2)	2.13	0.05	***						
A/swine/Italy/50127/2007 (H3N2)	1.45	0.10	***						
Human pandemic viruses									
A/Hong Kong/1/1968 (H3N2)	0.61	0.05	*	5.08	0.07	*			
A/Hamburg/05/2009	0.67	0.14	*	5.06	0.07	*			

*[a], Viral phenotypes were studied using three assays described in method paragraphs **3.3.5**, **3.3.6** and **3.3.9**. The data show mean values of at least two independent experiments and their 95% confidence intervals (CI). P values for the difference with respect to a representative avian virus (A/duck/Schleswig/21/1979) were calculated using unpaired two-tailed Student's t-test; *, P < 0.05; **, P < 0.005; ***, P < 0.0005.*

[b], All viruses are H1N1, if not indicated otherwise.

A strong sensitivity to NH_4Cl during cell entry can be observed for avian H1N1 virus infection ranging from 0.26 to 0.55 mM NH_4Cl (**figure 4.2a, table 2**). In contrast, swine viruses display a lower and broader range of NH_4Cl susceptibility varying between 0.6 and 2.5 mM. The two human viruses tested show a slightly higher susceptibility (0.77 mM) when compared to avian viruses but do not differ significantly. The ability of swine viruses to infect cells in the presence of higher NH_4Cl concentrations suggests that swine viruses enter the cell at a higher pH than both avian and human viruses.

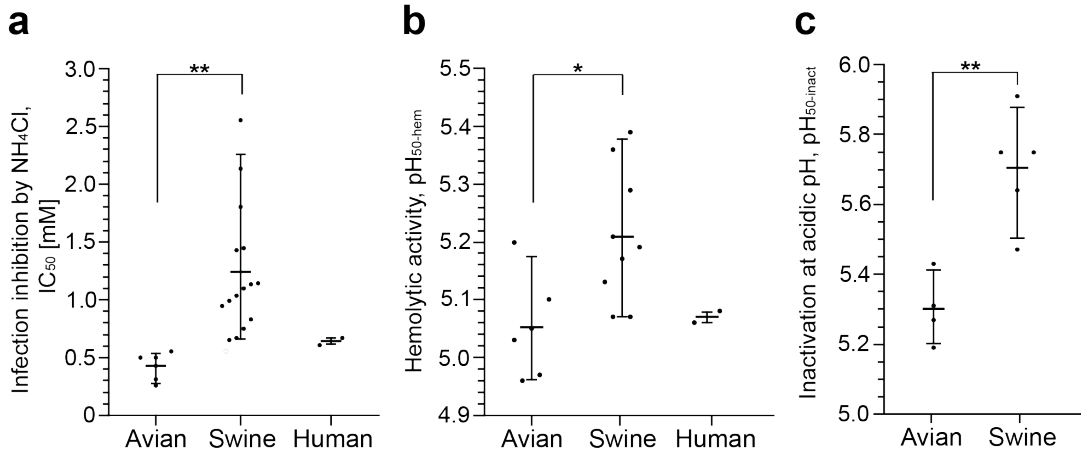

Figure 4.2: Membrane fusion activity and stability of H1N1 viruses. *Data depict species related mean values of IC_{50} of NH_4C (**a**), hemolytic activity ($pH_{50\text{-}hem}$) (**b**) and pH stability ($pH_{50\text{-}inact}$) (**c**) calculated from single experimental points in **table 2**. P values for the differences between viruses of birds, pigs and humans were calculated from single experimental points using two-sided unpaired Student´s t-test; *, P < 0.05; **, P < 0.005.*

In addition to pH-dependent fusion activity of the HA, properties of other viral proteins (M2, M1 and NP) may affect the pH optimum of virus entry into cells. M2 supports viral release regulating the intra-virion pH from that of the endosomal pH (reviewed in Scott and Griffin, 2015). The interaction between M1 and NP determines the dependence of RNP dissociation on acidification. Furthermore, interactions between M1 and HA/NA may affect fusion efficiency (reviewed in Edinger et al., 2014). In order to characterise the HA-specific pH dependency, HA hemolytic activity was determined. Virus induced lysis of human red blood cells (hRBCs) was quantified for different pH values. The pH of 50% hemolytic activity ($pH_{50\text{-}hem}$) was used for comparison (**table 2**). Mean values for viruses from different hosts are depicted in **figure 4.2b**. The pH of hemolytic activity for avian viruses lies within a narrow range between 4.9 and 5.2 pH units. (mean: pH 5.0) The HA of swine viruses confer erythrocyte hemolysis ranging from pH 5.1 to 5.4 (mean: pH 5.25). The tested human viruses display a pH optimum of hemolytic activity at pH 5.1 and thus are comparable to avian viruses. The viral

stability depends on the pH optimum of the HA conformational transition (see section **1.5.2**). In the absence of a target membrane the structural rearrangement resulting in the fusion competent conformation leads to viral inactivation due to abolished receptor-binding. To study the pH-dependent viral stability, avian and EAsw viruses were incubated in buffers with decreasing pH followed by MDCK cell infection. The pH at which viruses were inactivated by 50% ($pH_{50\text{-}inact}$) are listed in **table 2,** and mean values for avian and swine viruses were calculated accordingly (**figure 4.2c**). The pH that inactivates infection of avian viruses ranged from 5.2 to 5.5 (mean: pH 5.3). Porcine viruses are inactivated at a mean pH of 5.7 and pH values for single viruses ranged from 5.5 to 5.9. Consequently, the higher pH of infection inhibition of EAsw viruses suggests a decreased stability when compared to their avian precursors.
In agreement with the lower susceptibility to NH_4Cl during cell entry and the higher pH of hemolytic activity, this indicates that for swine HAs the conformational transition is initiated at a higher pH than for avian and human viruses. This likely allows swine viruses to infect target cells following a less strong acidification.

4.1.1 Analysis of HA sequences of avian and avian-like swine H1N1 influenza viruses

In order to identify amino acid substitutions in the HA that emerged during the establishment of the EAsw lineage and potentially affect membrane fusion activity and HA stability, HAs of all tested viruses were sequenced. The amino acid sequence at the nodes of the phylogenetic tree which separate EAsw from avian viruses (**figure 4.1a**) was reconstructed by comparing HAs of EAsw viruses isolated from 1979 to 1981 with all avian H1 HAs available to date. This comparison revealed eight amino acid differences between the putative first EAsw virus and the avian precursor (**table 3, figure 4.2**). HA sequence alignment of closely related avian and EAsw viruses used in this study is depicted in **figure 4.3**. Amino acids are numbered according to H1 HA numbering in **figure 4.3** and are expressed in accord to the H3 numbering system of Noubsawa et al. 1991, hereinafter (see **table 3**).

Table 3: Amino acids at H1 HA positions that separate Eurasian avian-like swine viruses from their putative avian precursor.[a]

	126a (138) [b]	**155 (169)**	**188 (202)**	**190 (204)**	**49_2 (393)**	**72_2 (416)**	**75_2 (419)**	**113_2 (457)**
Hypothetical avian precursor	S	T	A	E	T	N	R	S
Hypothetical first EAsw virus	N	I	T	D	S	D	K	F
Avian viruses [c]	N [285]	T [285]	T [370]	E [452]	T [452]		R [400]	S [454]
	S [167]	I [164]	V [53]	Q [2]	I [3]	N	K [54]	F [1]
	T [1]	V [4]	A [28]	X [1]			Q [1]	
	X [2]	X [2]	I [3]					

[a] *Sequences of the first EAsw virus and its avian precursor were inferred based on the HA sequences of avian and EAsw influenza viruses as described in the methods paragraph* ***3.4****. Amino acids are shown in a single letter code; X depicts ambiguity at this position. Numbers in square brackets reflect numbers of sequences with indicated amino acid.*

[b] *The first number is based on the H3 numbering system in accord with H3/H1 alignment of Nobusawa et al., 1991. A separate numbering is used for HA1 and HA2 subunits; the subscript refers to HA2. The number in brackets corresponds to the number of the codon within the complete coding sequence of the HA precursor (codons 1-17, signal peptide; codons 18-566, HA0).*

[c] *Analysis of 455 full-length H1 HA sequences.*

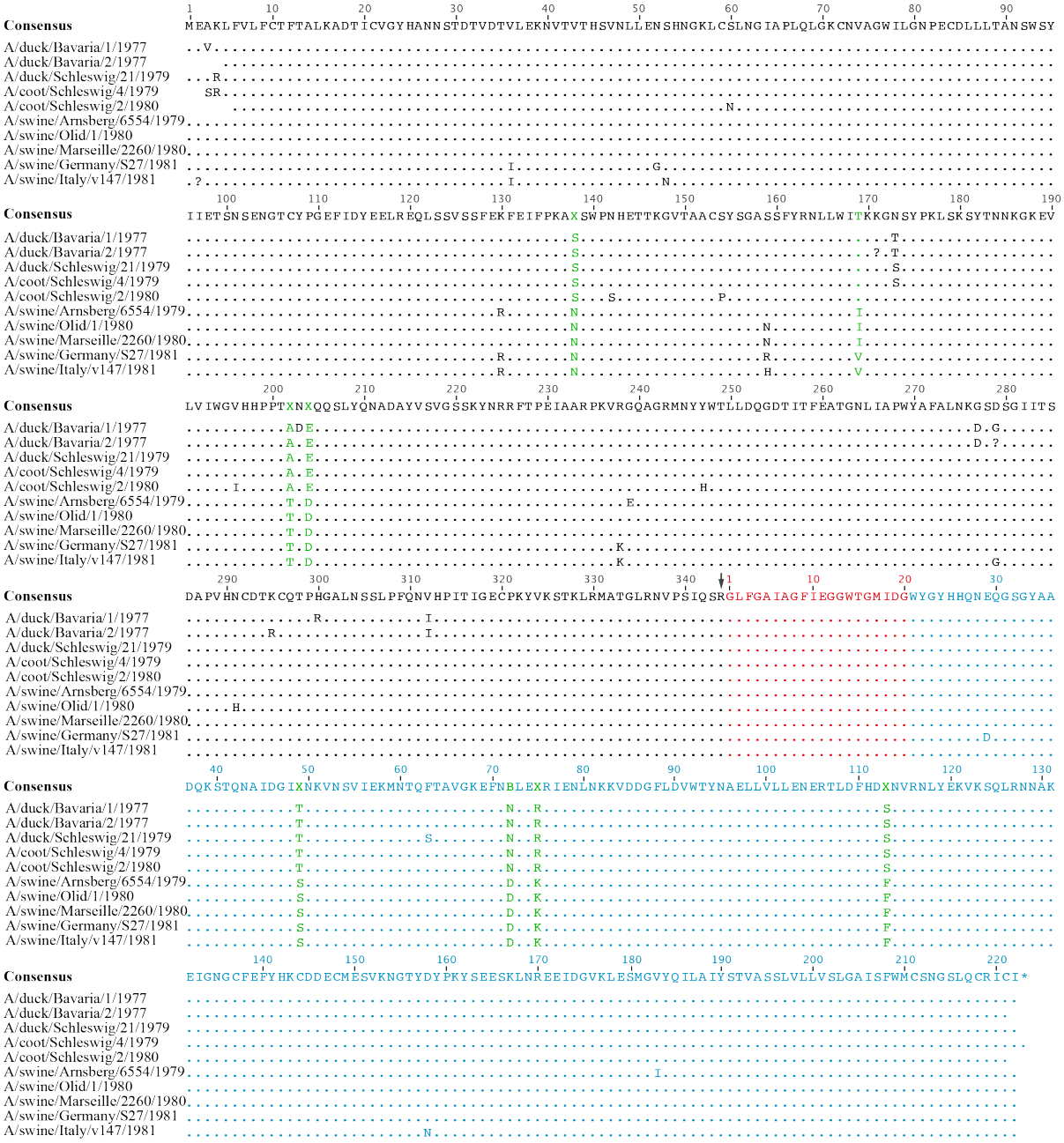

Figure 4.3: Comparison of amino acid sequences of closely related avian and EAsw viruses. *The HA0 cleavage site is indicated by an arrow. The fusion peptide and HA2 are coloured in red and blue, respectively. HA1 is numbered according H1 numbering. Numbering is restarted for HA2. Dots indicate sequence accordance with the consensus sequence. Amino acid motif that differ between all avian and swine virus HAs are depicted in green.*

Four substitutions are located in the globular head domain of the HA1 subunit (**figure 4.4a**; positions: 126a, 155, 188 and 190; H3 numbering). The two amino acid changes T155I and E190D are in close proximity to the receptor-binding pocket (**figure 4.4b**) and directly interact with sialic acid moieties of the receptors. The mutation E190D is known to facilitate adaptation of H1 viruses to α2,6-linked sialic acids in humans and pigs (Glaser et al., 2005; Matrosovich et al., 2000). Furthermore, glutamic acid at position 190 is conserved among all 16 HA subtypes

of aquatic bird viruses. The amino acid 188 is situated at the rim of the receptor-binding pocket whereas the conservative mutation S126aN is highly exposed to the solvent and is located in a 20 Å distance from the receptor-binding site. A potential influence of both residues on receptor-binding properties is unlikely but cannot be excluded. Remarkably, none of the four HA1 mutations are located in regions known to influence HA conformational change or stability.

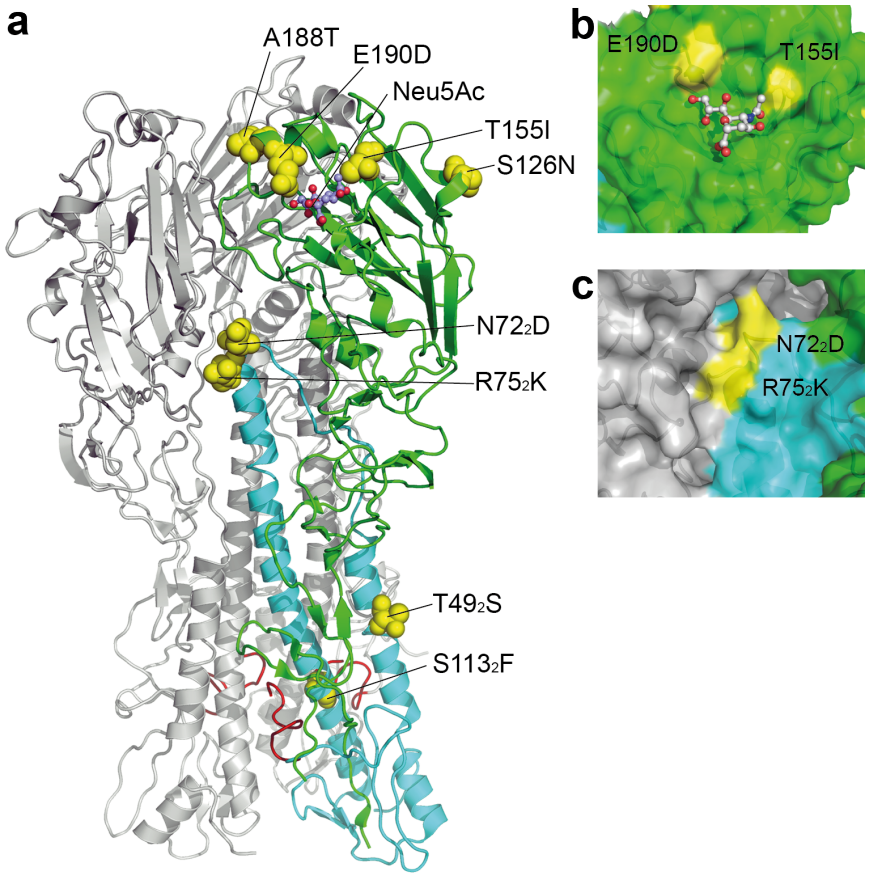

Figure 4.4: Amino acid differences between avian and early avian-like swine H1 HAs. a *The globular head domain of HA1 (green) contains four amino acid differences (yellow): S126N, T155I, A188T and E190D. Four additional substitutions (yellow) are located in the HA2 subunit (cyan): T49$_2$S, N72$_2$D, R75$_2$K and S113$_2$F. The fusion peptide is coloured in red. Amino acid positions are numbered according to H3 numbering. Mutations are illustrated in X-Ray resolved crystal structure of A/mallard/Alberta/35/1976 (2WRH, protein data bank).* ***b*** *Receptor-binding pocket with sialic acid receptor as ball-and-stick model.* ***c*** *Interface between two HA monomers in the region of the C helix to B loop transition (cyan).*

Another set of substitutions (T49$_2$S, N72$_2$D, R75$_2$K and S113$_2$F) is localized along the HA2 subunit (**figure 4.4**). The conservative substitution threonine to serine at amino acid residue 49$_2$ is located in the midsection of the small HA2 helix A and does not contact other parts of the HA molecule. Therefore, this substitution is unlikely to affect HA fusion properties. Positions 72$_2$ and 75$_2$ are part of the B loop structure connecting the large central helix C with

the antiparallel helix A. Both residues are in close contact with other HA monomers of the trimeric complex (**figure 4.4c**). Substitutions in this region are known to alter HA fusion properties (Mair et al., 2014; Russell, 2014; Skehel and Wiley, 2000). Amino acid 113_2 is located within the long central helix A of HA2 with side chains facing towards the internal hydrophobic pocket, which harbours the fusion peptide. The side chains contact phenylalanine of residue 3_2 within the fusion peptide and interact with leucine in position 2_2 of the neighbouring HA2 monomer. Amino acid exchanges influencing the fusion peptide pocket are described to alter HA fusion and stability properties (Mair et al., 2014; Russell, 2014; Skehel and Wiley, 2000). All of the analysed avian HA sequences harbour serine (S) at position 113_2, with one exception (Genbank accession number KC209515) that expresses phenylalanine (F) at this residue, similar to the EAsw HAs. Remarkably, this mutation emerged after several passages of an avian virus (A/mallard/Netherlands/10-Nmkt/1999 (H1N1)) in new born pig tracheal cells (Bourret et al., 2013). Hence, this mutation may represent adaptation to cells of the porcine respiratory tract.

Collectively, our analysis of the HA mutations separating avian and swine viruses suggests that one to three substitutions in the HA2 subunit may have contributed to the observed alteration in the pH requirements for viral cell entry and HA stability.

4.1.2 Influence of mutations acquired during avian-to-swine transmission on H1 HA fusion properties

In order to investigate the potential fusion-modulating effect of the four mutations identified in the HA2 subunit, fusion activity of single point mutants was studied using cell-expressed HA in two cell-based fusion assays. To ensure a high level of expression HAs were cloned in pCAGGS plasmids. Single amino acid exchanges were introduced in the HA of A/duck/Bavaria/1/1977. The HA of a typical EAsw virus (A/swine/Marseille/2260/1980) was included in this study for comparison.

First, the expression of the wild type and mutant HA plasmids in HeLa cells was tested using flow-cytometric analysis. HeLa cells were transfected with each plasmid and HA expression was detected using a polyclonal anti-H1 antibody (method section **3.3.10**). **Figure 4.5** shows FITC fluorescent histograms for mock-transfected (grey) in relation to plasmid-transfected cells (red). Percentage of cells showing higher fluorescence signals than mock-transfected cells was calculated and used as a measure of protein expression levels. The avian HA and the

mutants (49_2S, 72_2D, 75_2K and 113_2F) show similar HA expression levels, ranging from 70.5% to 79.2%. These results indicated that mutations did not significantly affect the levels of protein expression.

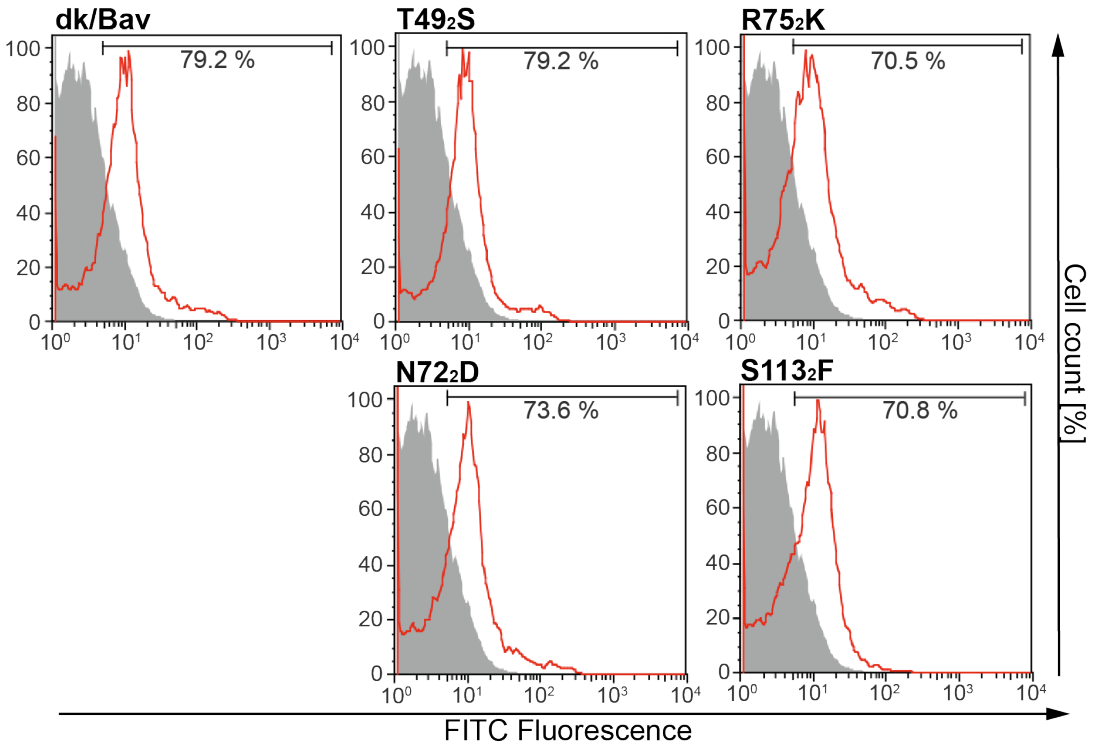

Figure 4.5: Flow-cytometric analysis of HA surface expression in HeLa cells. *HeLa cells were transfected with plasmids encoding wild type and mutant HAs. Surface expression was detected using rabbit anti-H1 and FITC-conjugated anti-rabbit antibodies. 10,000 events were counted and percentage of FITC-fluorescent cells was quantified (red line) compared to empty-plasmid transfected cells (grey).*

In a next step, the expression plasmids were used in two different cell-based fusion assays. Both assays are based on detection of syncytia formation either by quantification of microscope images or by measuring luciferase reporter gene expression.

In order to quantify syncytia formation by light microscopy, HA expressing HeLa cells were exposed to low pH conditions followed by Giemsa staining. The ratio of cell nuclei within syncytia and the total nuclei amount was calculated to define the pH of fusion induction (method section **3.3.11**). To determine syncytia formation using luciferase reporter gene expression HA expressing HEK293 cells were co-transfected with a *Renilla* luciferase coding plasmid (pGL4.73), and a plasmid coding T7-promotor driven *Firefly* luciferase (pTM-Luc). The cells were mixed with BHK-T7 cells constitutively expressing T7 polymerase. After low pH treatment *Renilla* and *Firefly* luciferase activity was measured. *Firefly* luciferase activity

was normalized to transfection efficiency represented by *Renilla* luciferase activity (method section **3.3.12**).

Using both assays (**figure 4.6** and **4.7**), the wild type HA of A/duck/Bavaria/1/1977 was observed to induce fusion starting at pH 5.4. The introduction of the avian-like swine amino acids in HA at position 72_2, 75_2 and 49_2 did not alter the pH of fusion induction when compared to the avian precursor. In contrast, 113_2F displayed a 0.2 pH units higher pH of fusion induction when compared to the avian HA. Microscopic images for the representative EAsw virus HA (A/swine/Marseille/2260/1980) show intense syncytia formation starting at pH 6. In contrast, *Firefly* luciferase activity shown by the porcine HA is not detectable down to pH 5.0 (**figure 4.7**). This may be a consequence of increased cell lysis during intense cell-to-cell fusion; resulting in reduced *Firefly* luciferase synthesis.

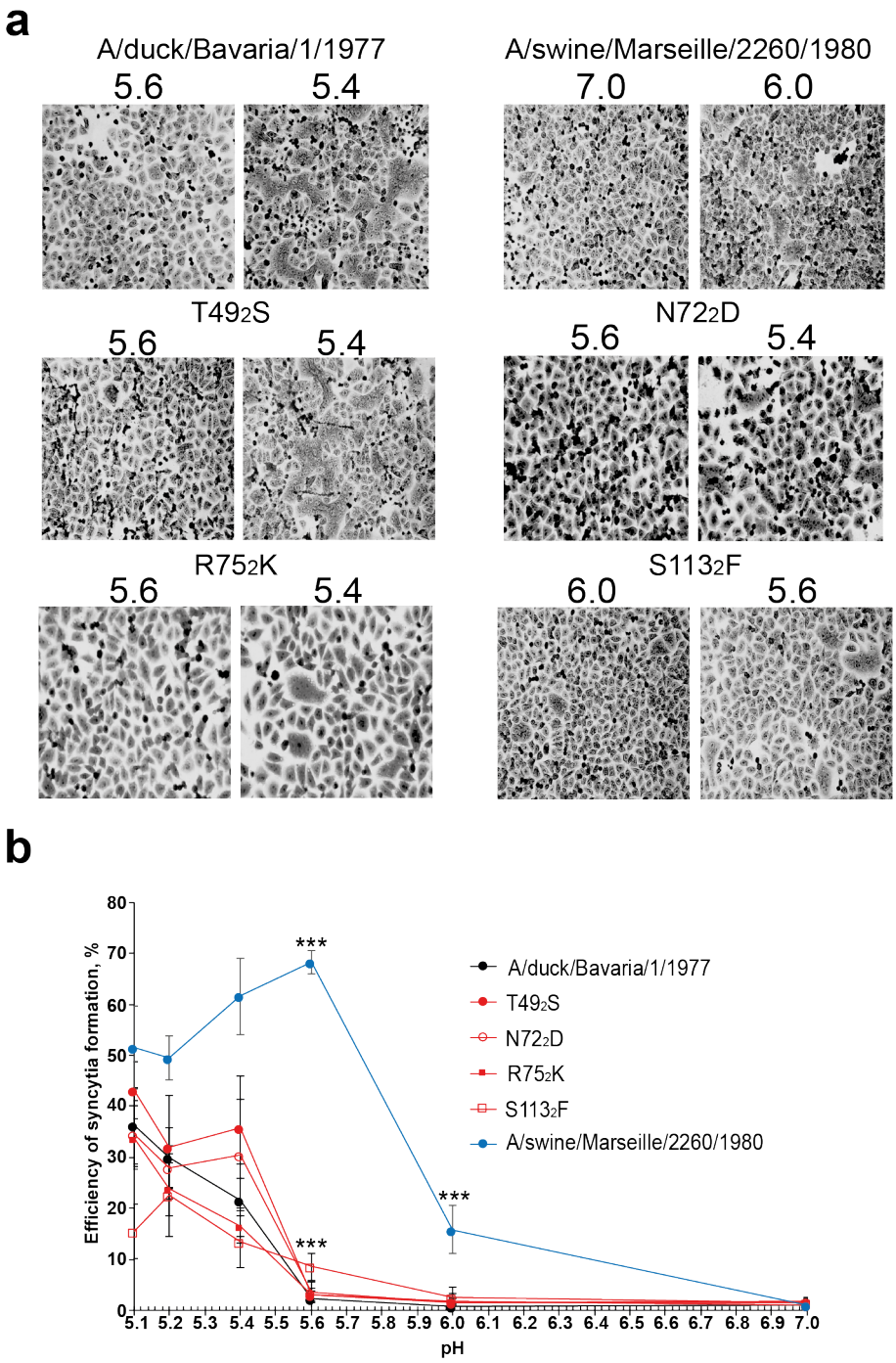

Figure 4.6: Syncytia formation at low pH in HeLa cells expressing wild type and mutant HAs. *HeLa cells were transfected with avian (black) or swine (blue) wild type or mutant HA (red). Syncytia formation was monitored over a range of pH 5.1 – 7.0* ***a*** *Representative images of fields at pH values where syncytia formation was observed first and images from the next higher used pH value are depicted. Images were taken at 300x magnification.* ***b*** *Efficiency of syncytia formation within the single fields was determined as percentage of cell nuclei in syncytia to total numbers of cell nuclei in the same microscopic field. Experiments were repeated twice independently on different days. P values were calculated using two-sided unpaired Student´s t-test; ***, $P < 0.0005$.*

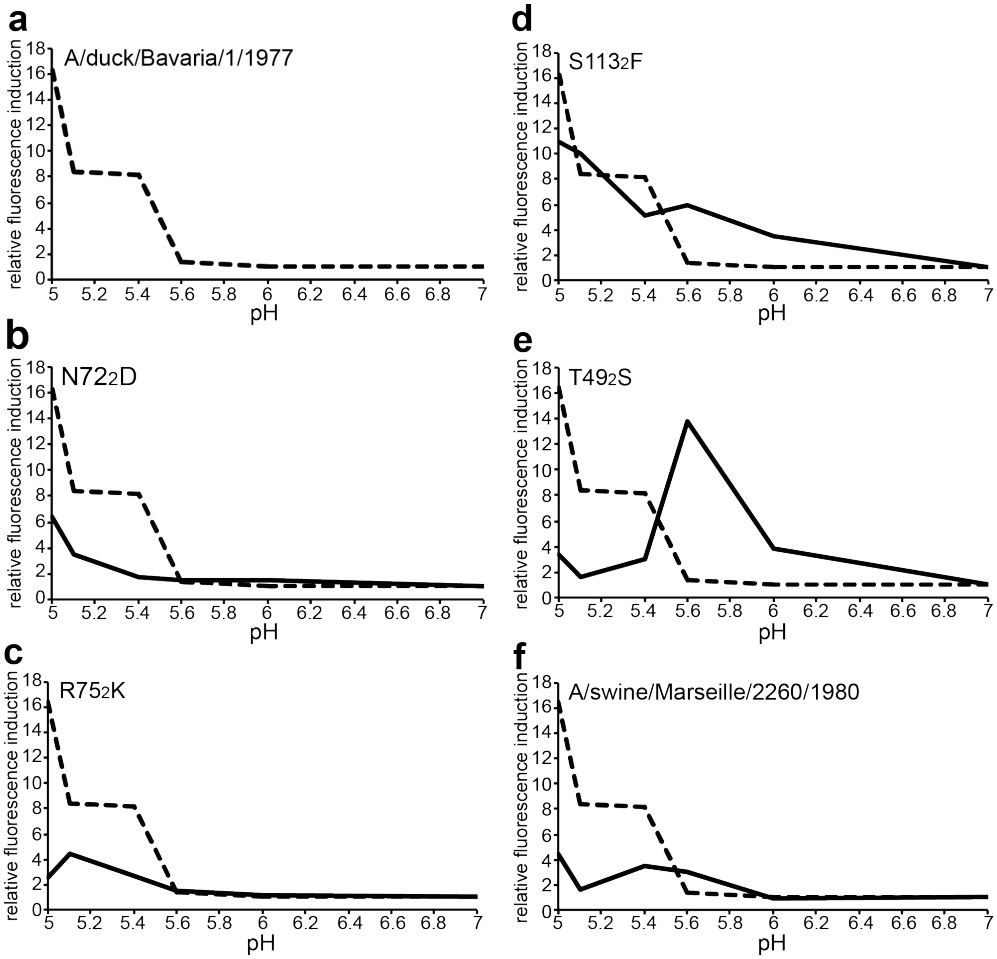

Figure 4.7: Luciferase activity of HEK293-BHK-T7 hybrid cells after HA cell-to-cell fusion. *HeLa cells were transfected wild type or mutant plasmid DNA, pGL4.73 and pTM-Luc. Syncytia formation efficiency was quantified by determining luciferase activity in relation to transfection efficiency (Renilla luciferase activity) at different pH values (5.0 – 7.0). Fluorescence values are plotted as relative induction compared to mock-transfection. Relative luciferase activity of avian wild type (**a** and dashed lines **b - f**), mutants (**b** – **e**; solid lines) and the representative swine HA (A/swine/Marseille/2260/1980, **f**; solid line) are depicted. Graphs show representative results from three independent experiments.*

In conclusion, the mutation $S113_2F$ identified during sequence comparison facilitates an increase in HA-mediated fusion, whereas effects of other three mutations introduced individually were below the detection limits of these assays.

4.1.3 Phenotypic characterisation of the HA of A/duck/Bavaria/1/1977 virus after serial passages in pigs.

The aim of this project was to identify adaptive mutations associated with avian-to-swine transmission. Subsequently, the nature of the mutations was to be studied. On this account,

receptor-binding and stability properties as well as replication efficiency in primary human tracheo-bronchial epithelial (HTBE) cells was investigated.

In cooperation with the Friedrich-Löffler Institute (FLI; Insel Riems, Germany) and the National Veterinary Research Institute (NVRI; Pulawy, Poland), the avian A/duck/Bavaria/1/1977 (H1N1), a putative precursor of EAsw viruses, was serially passaged in pigs. For this purpose, pigs were infected intranasally and lung tissue samples were collected four days post infection. Sentinel pigs were infected with tissue homogenates prepared from collected pooled samples. Four days post infection the procedure was repeated. After passage 0, 5, 10 and 15 sentinel contact pigs were introduced 2 days post infection in order to monitor transmission efficiency (Van Reeth, 2014). Colleagues at the FLI and NVRI isolated viruses after passage 15 and 19, respectively. Whereas the original duck virus did not transmit by direct contact in pigs, both passaged isolates displayed limited swine-to-swine transmission.

The HAs of both pig-passaged viruses were sequenced. The sequence analysis revealed five coding mutations within the HA glycoprotein of A/duck/Bavaria/1/1977 isolated at the FLI (dk/Bav p15): L49M, T155I, K166A, V178I and Q226L (H3 numbering). The virus obtained at the NVRI (dk/Bav p19) displayed four amino acid substitutions: K166E, E190D and G225E in HA1 subunit and $G67_2S$ in the HA2 subunit (**figure 4.8a**).

The substitutions E190D, Q226L and G225E (**figure 4.8b,c**) are part of the receptor-binding site and are well known to facilitate a switch in the receptor-binding specificity from avian to human type preference (Matrosovich et al., 2000, 1997). The side chain of the amino acid at position 155 participates in the formation of the pocket that accommodates the acyl substituent at 5-N of Neu5Ac. Mutations in this position occurred independently both in the classical swine and avian-like swine viruses and it was assumed that these mutations serve to increase the affinity of the virus for 5N-glycolyl analog of the sialic acid that is abundant in pigs but absent in birds and humans (Matrosovich et al., 2000). Glutamic acid at position 190, glycine at position 225 and glutamine at position 226 are conserved among avian viruses of 16 HA subtypes (Matrosovich et al., 2008).

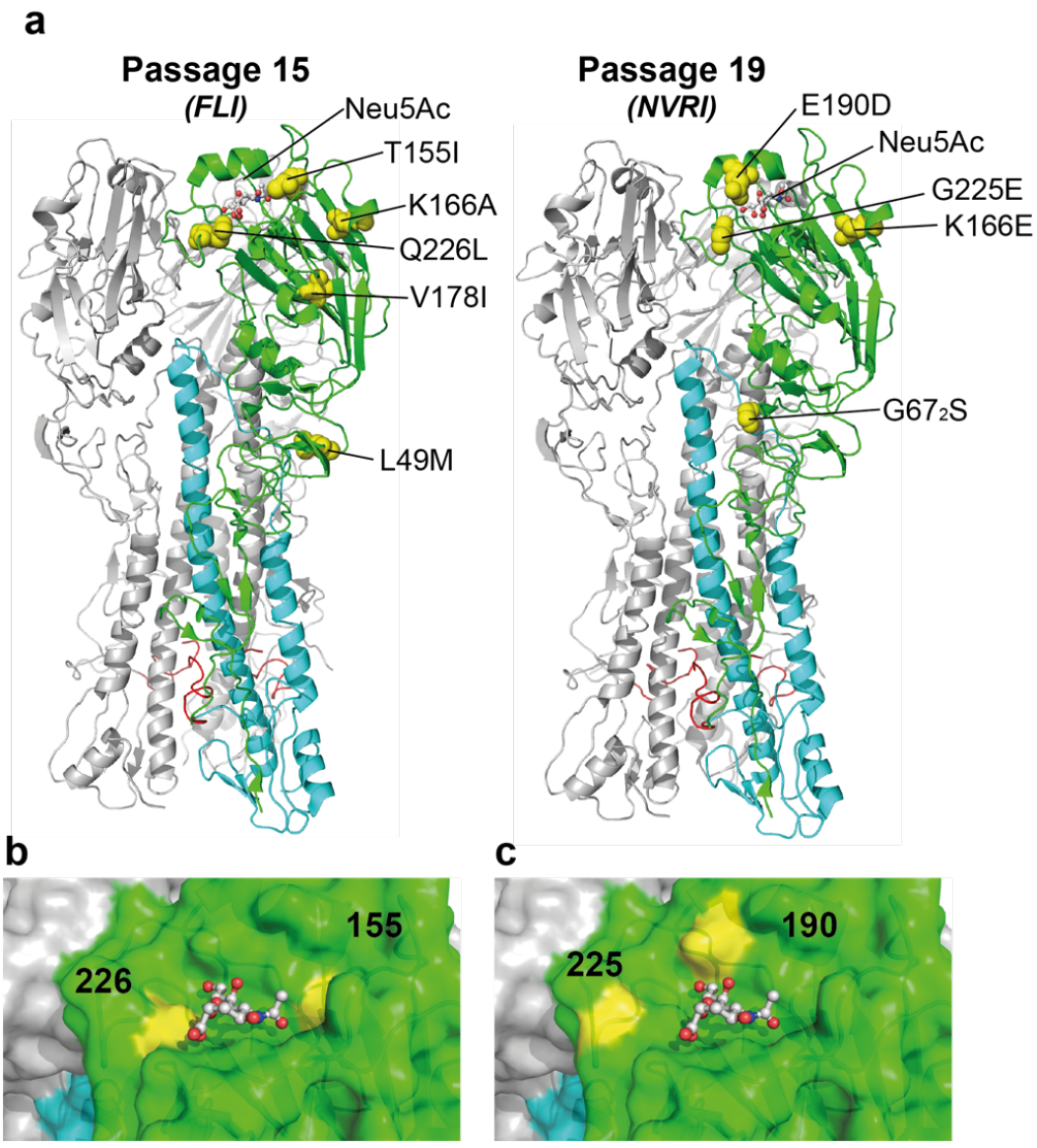

Figure 4.8: Location of mutations in the HA acquired during passaging of A/duck/Bavaria/1/1977 in pigs. ***a*** *One HA monomer is shown in green (HA1) and cyan (HA2). The fusion peptide is coloured in red. Mutations identified in the HA of two pig-passaged viruses dk/Bav p15 and dk/Bav p19 are illustrated using X-ray data of H1 HA (2WRH, protein data bank). Amino acid positions are named according to H3 numbering.* ***b, c*** *Mutations (yellow) identified near the receptor-binding pocket. Sialic acid receptor is shown as ball-and-stick model.*

Substitution E190D is known to be essential for adaptation of H1 HA to α2,6-linked sialic acid receptors in humans and pigs and occurred several times in nature during pig adaptation. (Glaser et al., 2005; Matrosovich et al., 2000). Interestingly, both adaptation experiments resulted in amino acid substitutions at position 166 which is located within the globular head domain distal to the receptor-binding site. Whereas for dk/Bav p19 (NVRI) lysine (K) was substituted by glutamic acid (E), dk/Bav p15 (FLI) displayed a substitution by alanine (A). The

resulting decrease in positive charge of the globular head may influence receptor-binding avidity.

Each virus acquired one mutation within the HA stalk; L49M (dk/Bav p15) and G67$_2$S (dk/Bav p19) respectively. The mutations are located in the HA stalk and are likely involved in the HA conformational transition during fusion following acidification and potentially influences HA-stability.

4.1.3.1 Alteration in receptor-binding specificity during pig passaging

One early step during adaptation to pigs is a switch in receptor-binding specificity (section **1.5.1.1**) from avian (α2,3) to human type (α2,6). The receptor preference, therefore, can be used to estimate the degree of adaptation. In order to monitor ongoing adaptation receptor-binding specificity of the pig-passaged viruses should be investigated. Both viruses acquired at least two mutations within the HA that are associated with alterations of receptor-binding specificity (dk/Bav p15: Q226L and T155I; dk/Bav p19: G225E and E190D). To characterise potential changes in the receptor-binding properties, binding to HRP-conjugated fetuin, exhibiting either α2,3- or α2,6-linked sialic acid moieties, was studied. Virus-bound HRP-fetuin was quantified using TMB and the absorption was measured at 450 nm. Association constants (K_{ass}) of virus-fetuin complexes were calculated as described in **3.3.13**.

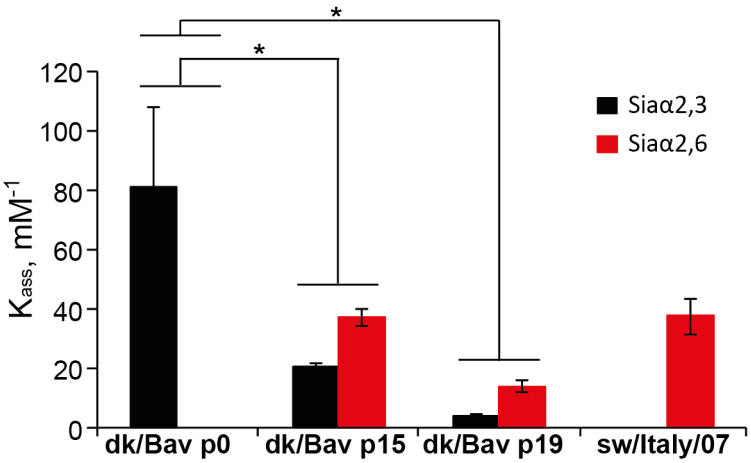

Figure 4.9: Receptor-binding of the original avian virus and its pig-passaged variants. *Association constants (K_{ass}) for HRP-conjugated fetuin possessing either α2,3- (black) or α2,6-linked (red) sialic acid moieties were calculated for avian wild type dk/Bav p0, the two pig-passaged viruses dk/Bav p15 and dk/Bav p19 and a representative swine virus (sw/Italy/07). Experiments were performed twice independently in duplicates each. P values were calculated using two-sided unpaired Student´s t-test; *, P < 0.05.*

The avian wild type virus displayed exclusive binding to sialic acids linked to the penultimate sugar by a α2,3 linkage (**figure 4.9**). Upon several passages in pigs the binding preference of dk/Bav p15 and dk/Bav p19 changed to predominant recognition of α2,6-linked sialic moieties, although weak binding to avian type receptors remains. A fully adapted representative swine virus (sw/Italy/07) possess binding to α2,6-linked sialic acids exclusively. The data indicate that the pig-passaged avian A/duck/Bavaria/1/1977 viruses acquired human type receptor-binding affinity.

4.1.3.2 Changes in HA stability during adaptation of A/dk/Bavaria/1/1977 in pigs

In section **4.1** it could be demonstrated that HA stability changes during natural avian-to-swine transmission. On that account it was to be investigated whether dk/Bav p15 and dk/Bav p19 differ in HA stability from their avian precursor after experimental adaptation. Both pig-passaged viruses acquired HA mutations that potentially influence pH induced fusion and HA stability (L49M in dk/Bav p15 and G67$_2$S in dk/Bav p19). In order to investigate viral stability under low pH conditions the viruses were exposed to low pH environments, followed by MDCK cell infection (see **4.1**).

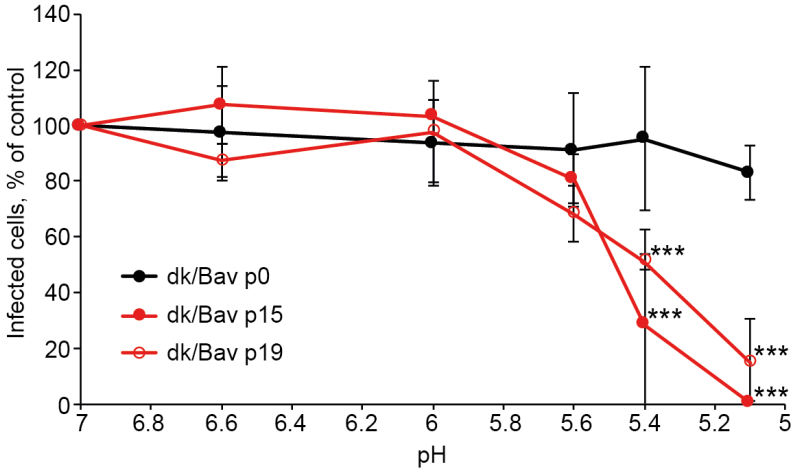

Figure 4.10: Acid stability of two pig-passaged viruses (dk/Bav p15 and dk/Bav p19, red) and their avian precursor A/duck/Bavaria/1/1977 (black). *Remaining infectivity after low pH treatment was determined using focus forming assay in MDCK cells and expressed as percentage of infectivity under neutral conditions. Experiments were done in triplicates and values were obtained from two independent experiments. P values were calculated using unpaired two-sided Student´s t-test; ***, P < 0.0005.*

The infectivity of the avian precursor A/duck/Bavaria/1/77 showed no reduction (**figure 4.10**) down to pH 5.1 (90% infection). In contrast, pig-passaged viruses dk/Bav p15 and dk/Bav p19 revealed a significantly reduced ability to infect cells after incubation at pH 5.2 (30% and 60%, respectively) and 5.1 (0% and 10%, respectively). Therefore, both passaging experiments resulted in viruses exhibiting a decreased stability under low pH conditions. The result agrees with the analysis of natural avian and swine isolates and suggests a reduction in HA stability during virus adaptation to pigs.

4.1.3.3 Replication efficiency of pig-passaged A/duck/Bavaria/1/1977 in human tracheo-bronchial epithelial cells

Pigs are believed to be intermediate hosts that pre-adapt avian viruses for efficient replication in humans. Consistently, swine viruses are able to efficiently infect cells of the human airway epithelium. To test whether passaging in pigs increased the ability of the avian virus to replicate in humans, growth of the pig-passaged viruses in human tracheo-bronchial epithelial (HTBE) cultures was studied. HTBE cultures differentiate into ciliated and non-ciliated cells following growth on air liquid interface and serve as a model for human airway epithelium (Ilyushina et al., 2012; Matrosovich et al., 2007, 2004). Differentiated HTBE were infected with the avian wild type (dk/Bav 0) and the pig-passaged viruses dk/Bav p15 and dk/Bav p19. A swine virus (A/swine/Italy/50175/2007) and a human 2009 pandemic isolate (A/Hamburg/05/2009) were included for comparison. The viral growth was monitored for 144 h, and titres were determined as focus forming units (method section **3.3.2**).

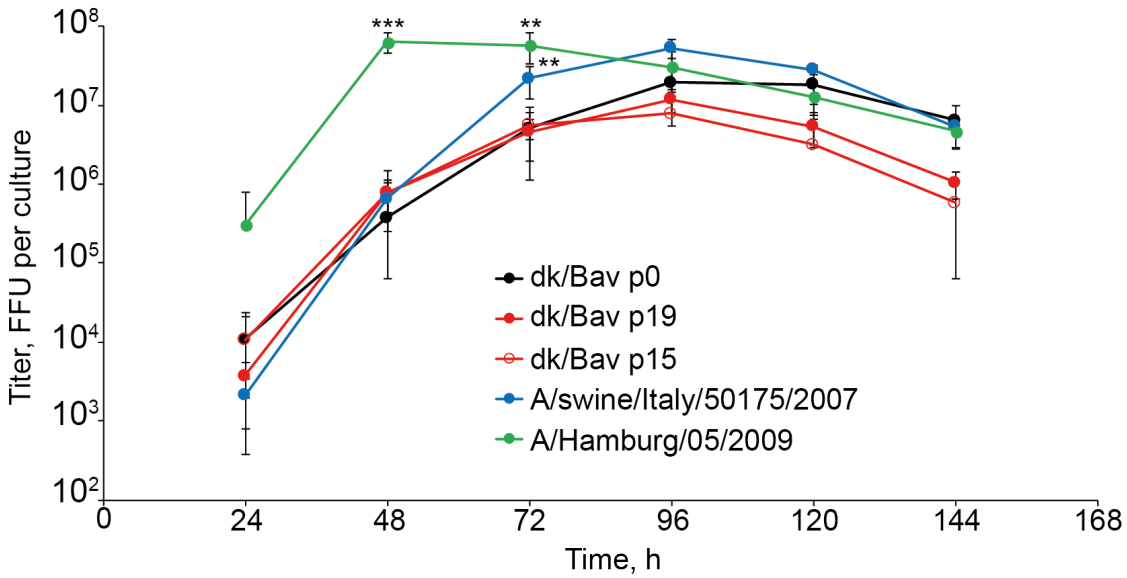

Figure 4.11: Growth curves of pig-passaged viruses (dk/Bav p15 and dk/Bav p19) and their avian precursor A/duck/Bavaria/1/1977 in HTBE culture. *Air liquid cultures of differentiated HTBE cells were infected with 10^4 FFU/ml of the pig-passaged viruses (dk/Bav p15 and dk/Bav p19, red), the avian precursor (dk/BAV p0, black), a swine virus (A/swine/Italy/50175/2007, blue) and a pandemic human isolate (A/Hamburg/05/2009, green). Viruses were isolated from the apical cell sides at indicated times, and titrated using focus forming assay. Mean values and standard deviations were calculated for 5 replicates cultures per virus. P values were determined using two-sided, unpaired Student´s t-test; **, $P < 0.005$; ***, $P < 0.0005$.*

The human pandemic virus A/Hamburg/05/2009 displayed fast growth kinetics reaching peak titres of 10^7 FFU after 48 h (**Figure 4.11**). The titre for the swine virus peaked at 10^7 FFU after 72 h, and consequently displayed a delayed replication efficiency when compared to the human virus. The wild type avian virus and the two pig-passaged viruses did not differ in their replication and reached titres of 10^6 FFU within 72 h.

On this account, the fully adapted swine virus replicates better than avian virus, but less effective than the pandemic virus. However, both pig-passaged viruses do not show significantly increased fitness in human airway epithelium.

In sum, the pig-passaged viruses acquired human type receptor-binding as observed for swine viruses. In agreement with natural avian-to-swine transmission, experimental adaptation to pigs resulted in a decreased stability. These changes do not seem to be sufficient to mediate efficient replication in the human respiratory tract.

4.2 Fusiogenic activity and stability of H7 viruses from different avian species

Recent studies indicate that HA stability and pH of fusion changes during transmission between avian and mammal species. This work proved this for an avian-to-swine transmission event (section **4.1**). However, little is known about differences in stability and fusion in different bird species. Gianneccini and colleagues suggest that HA stability and membrane fusion properties alter during transmission between bird species, too (Giannecchini et al., 2006). Nevertheless, this study comprised only a limited number of viruses and a systematic study of membrane fusion and stability properties are missing.

To fill this gap in knowledge fusion properties of H7 viruses isolated from several wild bird species and from terrestrial poultry were to be compared. Our lab had previously compared receptor-binding properties of a large set of H7 viruses including representatives of all major H7 lineages (Gambaryan et al., 2012). This panel provides the opportunity to test fusion properties of wild bird and poultry viruses. In total, 13 wild bird viruses and seven poultry viruses were selected (**Figure 4.12**) from two distinct H7 lineages; the Eurasian (EA) and the North American (NAm) lineage. The characterisation of viral fusion properties was performed in part by Nancy Mounogou Kouassi in the course of her master thesis (Mounogou Kouassi, 2014).

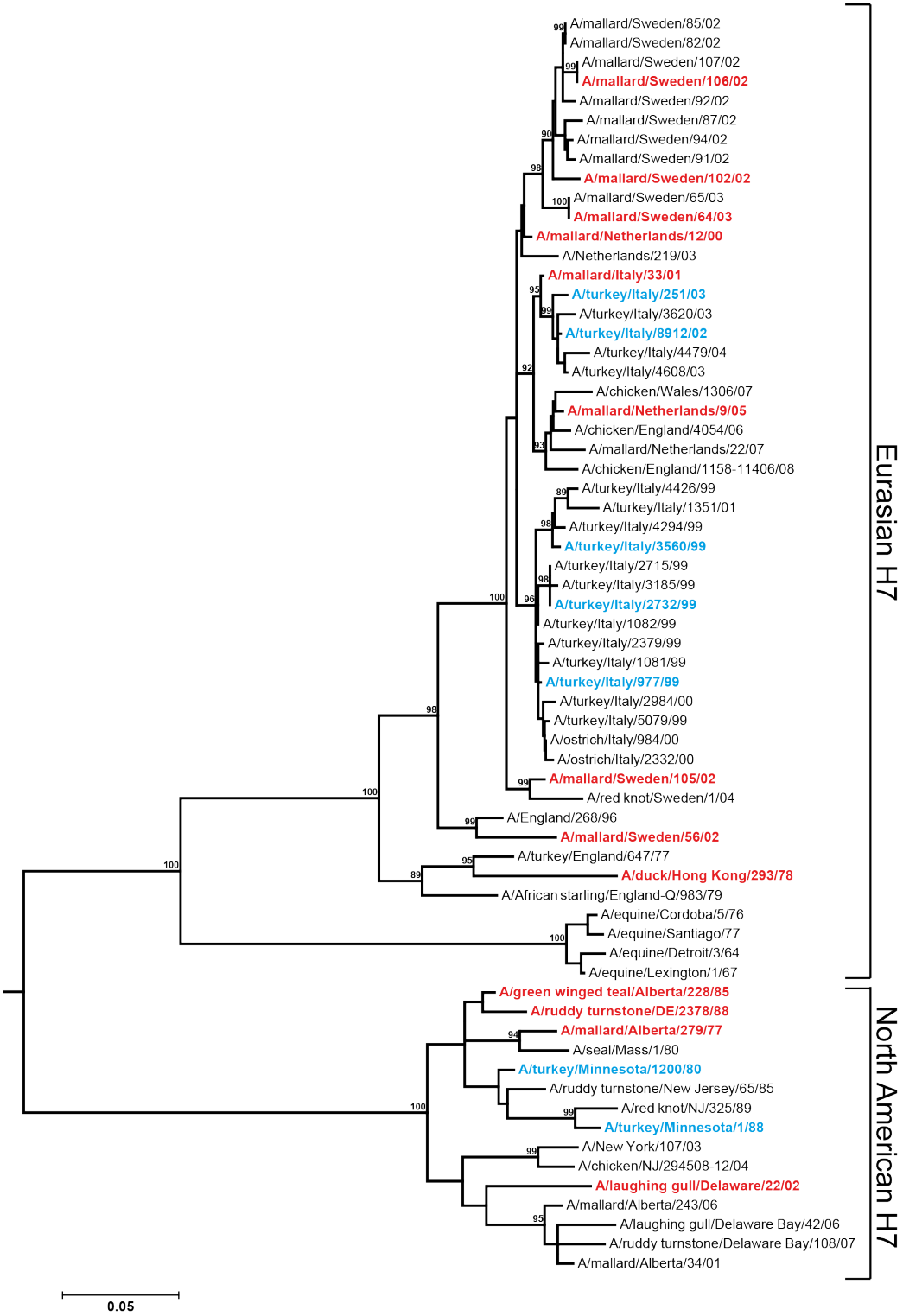

Figure 4.12: Phylogenetic relationship of avian H7 viruses. *The evolutionary tree for HA amino acid sequences was generated using the neighbour-joining method included in MEGA6 (Tamura et al., 2013). Statistical analysis was performed by bootstrapping (100 cycles). The tree based on sequences of 65 H7 subtype viruses (for accession numbers see* ***2.11****) available in our lab (Gambaryan et al., 2012). The scale bar represents 0.05 amino acid substitutions per site. Viruses used in this study are coloured in red (wild bird viruses) and blue (poultry viruses).*

To investigate HA-mediated fusion and viral stability of wild bird (nine EA and four NAm) and poultry H7 (five EA and two NAm) viruses, susceptibility to ammonium chloride, pH of hemolytic activity and acid stability was determined as described above (see section **3.3.5, 3.3.6, 3.3.9** and **4.1**). To compare H7 viruses with viruses from other subtypes, representative viruses from eight additional subtypes (H1, H2, H3, H4, H5, H13, H14, H16; section **2.11**) were included in this study. The individual experimental results are summarized in **table 4** and illustrated in **figure 4.13**.

Table 3: Fusion and stability properties of wild bird and poultry H7 viruses.

Virus[a]	Infection inhibition by NH4Cl, IC_{50} [mM]		Hemolytic activity, $pH_{50\text{-}hem}$		Inactivation at acidic pH, $pH_{50\text{-}inact}$	
	Mean	± CI	Mean	± CI	Mean	± CI
North American wild aquatic bird viruses						
A/mallard/Alberta/279/1977 (H7N3)	0.38	0.03	5.13	0.16	5.08	0.05
A/green winged teal/Alberta/228/1985 (H7N3)	0.66	0.08	5.07	0.03	5.17	0.03
A/ruddy turnstone/DE/1378/1988 (H7N7)	0.41	0.13	5.08	0.05		
A/laughing gull/DE/22/2002 (H7N3)	0.46	0.04	5.33	0.05		
Eurasian wild aquatic bird viruses						
A/duck/HK/293/1978 (H7N2)	0.383	0.021	5.03	0.05	5.03	0.05
A/mallard/Netherlands/12/2000 (H7N3)	0.98	0.17	5.03	0.05	5.28	0.03
A/mallard/Italy/33/2001 (H7N3)	0.60	0.14	5.00	0.06	5.16	0.016
A/mallard/Sweden/56/2002 (H7N7)	1.09	0.09	5.15	0.06	5.14	0.016
A/mallard/Sweden/102/2002 (H7N7)	1.22	0.07	5.08	0.05	5.43	0.03
A/mallard/Sweden/105/2002 (H7N7)	1.55	0.29	5.35	0.07	5.44	0.04
A/mallard/Sweden/106/2002 (H7N7)	0.300	0.019	5.03	0.05	5.15	0.000
A/mallard/Sweden/64/2003 (H7N7)	0.97	0.09	5.08	0.05	5.18	0.022
A/mallard/Netherlands/9/2005 (H7Nx)	0.78	0.07	5.12	0.03	5.45	0.05
North American H7 poultry viruses						
A/turkey/MN/1200/1980 (H7N3)	0.80	0.07	5.23	0.25	5.23	0.05
A/turkey/MN/1/1988 (H7N9)	0.66	0.05	5.20	0.20	5.08	0.05
Eurasian H7 poultry viruses						
A/turkey/Italy/977/1999 (H7N1)	1.17	0.24	6.2	0.7	5.53	0.07
A/turkey/Italy/2732/1999 (H7N1)	1.208	0.016	5.22	0.12	5.22	0.06
A/turkey/Italy/3560/1999 (H7N1)	1.97	0.12	6.7	0.6	6.09	0.18
A/turkey /Italy/8912/2002 (H7N3)	1.41	0.13	6.7	0.4	5.49	0.016
A/turkey/Italy/251/2003 (H7N3)	1.19	0.16	6.1	1.3	5.53	0.07
Viruses from other subtypes						
A/mallard/Alberta/119/1998 (H1N1)	0.42	0.07	5.15	0.10		
A/mallard/Alberta/205/1998 (H2N9)	0.48	0.12	5.35	0.098		
A/mallard/Alberta/290/1998 (H3N8)	0.252	0.003	5.08	0.049		
A/mallard/Alberta/47/1998 (H4N1)	0.58	0.13	5.23	0.182		
A/duck/Minnesota/1525/1981 (H5N1)	0.42	0.07	5.12	0.033	5.40	0.06
A/gull/Netherlands/04/2007 (H13N6)	0.32	0.03	5.13	0.049	5.42	0.03
A/mallard/Guryev/263/1982 (H14N5)	0.46	0.03	5.22	0.163		
A/gull/Netherland/01/2007 (H16N3)	0.43	0.09	5.33	0.049	5.47	0.03

*[a], Viral phenotypes were studied using three assays described in method paragraphs **3.3.4**, **3.3.5** and **3.3.8**. The data show mean values of at least 2 independent experiments and their 95% confidence intervals (CI).*

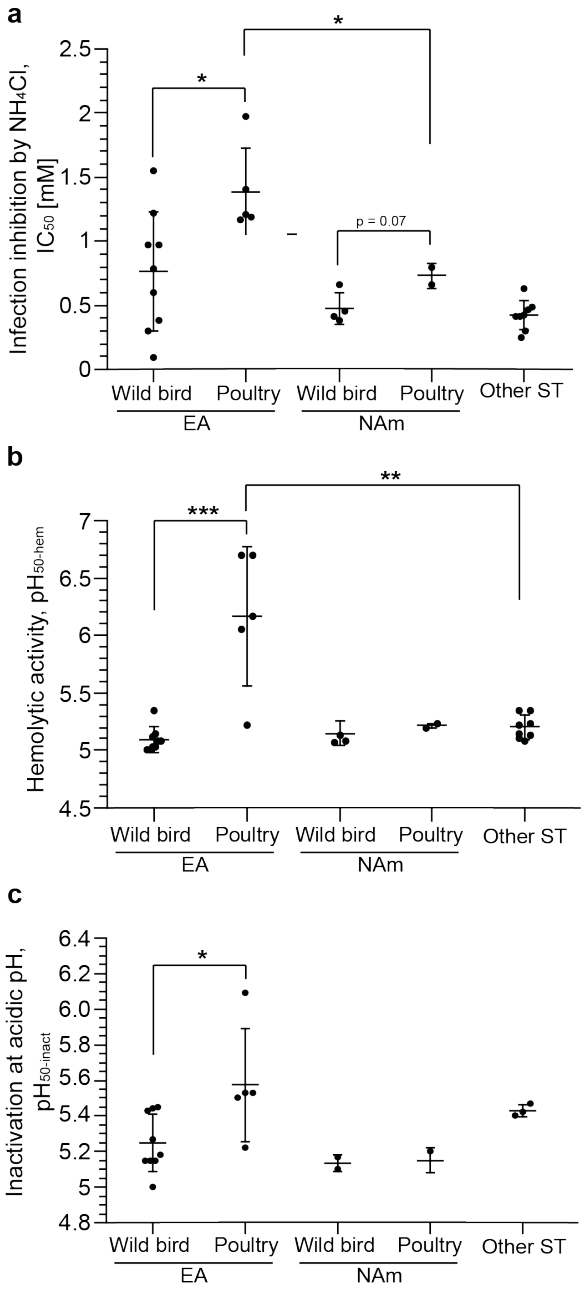

Figure 4.13: Membrane fusion activity and stability of EA and NAm H7 viruses. *Data depict species related mean values of IC_{50} of NH_4Cl (**a**), hemolytic activity ($pH_{50\text{-}hem}$) (**b**) and pH stability ($pH_{50\text{-}inact}$) (**c**) from the **table 4**. EA, Eurasian; NAm, North American; ST, subtypes. P values for the differences between viruses of birds, pigs and humans were calculated from single experimental points using two-sided unpaired Student´s t-test; *, P < 0.05, **, P < 0.005 ***, P < 0.0005*

During infection inhibition EA wild bird viruses show higher susceptibility to NH_4Cl (mean IC_{50}: 0.8 mM NH_4Cl) when compared to EA poultry viruses (mean IC_{50}: 1.4 mM NH_4Cl; **figure 4.13a**). In contrast, NAm wild bird viruses are not significantly stronger inhibited (mean: 0.5 mM NH_4Cl) than NAm poultry viruses (mean: 0.7 mM NH_4Cl). Additionally, EA H7 viruses from both species were less susceptible to NH_4Cl when compared to NAm H7 viruses and viruses from other subtypes.

In a next step, HA hemolytic activity of these viruses was investigated. The quantification of 50% lysis of red blood cells ($pH_{50\text{-}hem}$) was performed as described in the methods section (section **3.3.9**) and in the previous chapter (**4.1**). No difference was observed between NAm duck and NAm poultry viruses, both display $pH_{50\text{-}hem}$ of 5.1. (**figure 4.13b**). EA poultry viruses show an about 1 pH unit higher mean $pH_{50\text{-}hem}$ (mean $pH_{50\text{-}hem}$: pH 6.2) when compared to EA duck viruses (mean $pH_{50\text{-}hem}$: pH 5.2). Compared to NAm H7 and viruses from other subtypes (mean $pH_{50\text{-}hem}$: pH 5.1), EA poultry viruses display a significantly higher $pH_{50\text{-}hem}$.
Finally, acid stability of viruses was compared as described in **3.3.6** and **4.1**. As expected, EA poultry viruses display a 0.4 pH units higher pH of inactivation (mean pH_{inact}: 5.6) when compared to EA duck viruses (**figure 4.13c**). North American viruses display no significant difference between poultry (mean pH_{inact}: pH 5.2) and wild bird (mean pH_{inact}: pH 5.2) viruses. Viruses of other subtypes displayed a lower acid stability (mean pH_{inact}: pH 5.4) than viruses from EA poultry viruses.
Our results suggest that EA poultry H7 viruses are less dependent on acidification during cell entry, show a higher pH of hemolytic activity, and are less stable in acidic environment than EA duck H7 viruses. Furthermore, EA poultry H7 viruses show a higher pH of fusion and a decreased stability when compared to viruses of other subtypes. In contrast, a few NAm virus strains tested do not differ significantly from aquatic bird viruses of other subtypes.

4.3 Characterisation of human adapted influenza A viruses

Human infection with avian or swine viruses are typically restricted to individuals (reviewed in Freidl et al., 2014; Short et al., 2015). Recent reports suggest that membrane fusion properties contribute to limited transmission upon zoonotic infections. Unlike swine viruses (section **4.1**) human adapted seasonal and pandemic viruses possess a low pH of fusion induction accompanied by high stability (DuBois et al., 2011; Galloway et al., 2013; Reed et al., 2010; Scholtissek, 1985). Avian viruses differ in membrane fusion activity depending on subtype and strain. In order to identify changes in fusion during human infection, fusion properties of pandemic and zoonotic human HAs was to be investigated.

4.3.1 HA-mediated fusion of pandemic viruses

In order to study the pH of HA-mediated fusion of human adapted viruses, fusion induction of representatives from the four known pandemic viruses was characterised. The HAs of A/Brevig Mission/1/1918 ('Spanish flu'), A/Singapore/1/1957 ('Asian flu'), A/Hong Kong/1/1968 ('Hong Kong flu') and A/Hamburg/05/2009 ('Swine flu') were cloned into expression vectors (method section **3.1.4 - 3.1.6**) and the start of membrane fusion was investigated using a syncytia formation assay (method section **3.3.11**). In order to compare fusion properties of pandemic viruses with avian and swine viruses, HAs from A/duck/Bavaria/1/1977 and A/swine/Marseille/2260/1980 were included in the study.

Following incubation at low pH, the three pandemic HAs from A/Brevig Mission/1/1918, A/Singapore/1/57 and A/Hong Kong/1/1968 display fusion induction at pH below pH 5.2 (**figure 4.14**). The HA of the recent pandemic virus, A/Hamburg/05/2009, induces fusion at pH 5.4 and with this at the same pH as the avian HA of A/duck/Bavaria/1/1977 (**figure 4.14**). The representative porcine HA (A/swine/Marseille/2260/1980) promotes syncytia formation starting from pH 6 (**figure 4.14**).

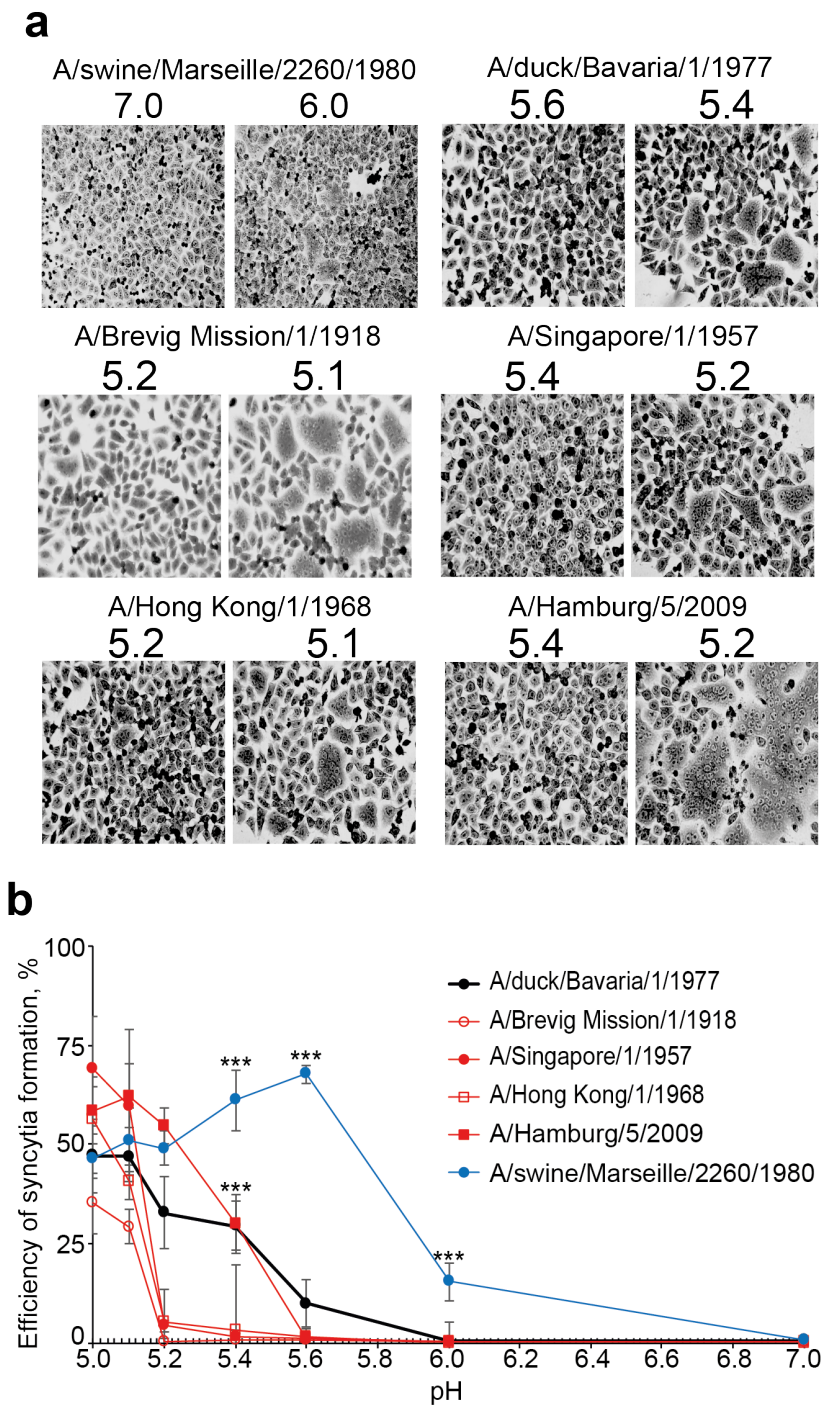

Figure 4.14: Syncytia formation in HA-expressing HeLa cells upon acidification. *HeLa cells were transfected with pandemic, avian or swine virus HA. Syncytia formation was monitored over a range of 5.1 – 7.0* ***a*** *Representative images of visual fields at pH values where syncytia formation was observed first and images from the next higher measured pH value are depicted. Images were taken at 300x magnification* ***b*** *Efficiency of syncytia formation within the single fields was determined as percentage of cell nuclei in syncytia to total numbers of cell nuclei in the same field. Values represent the mean of two independent experiments. P values were calculated using two-sided unpaired Student´s t-test; ***, $P < 0.0005$.*

In summary, the HAs of three human pandemic viruses isolated in 1918, 1957 and 1968 show a lower pH optimum of HA-mediated fusion than the tested avian and swine HAs. The 2009 pandemic HA initiate fusion starting from a 0.2 units higher pH when compared to the other pandemic HAs. This difference may be a consequence of the porcine origin of this virus and suggest this isolate, obtained early during the pandemic course, is not fully adapted to humans, yet.

4.3.2 Role of HA substitutions emerged in 1968 pandemic influenza virus

Adaptation of H5N1 viruses to ferrets suggest that at least four substitutions in avian HA may be required to establish transmission in mammals. These mutations alter receptor specificity, receptor-binding avidity, HA glycosylation and HA stability (Herfst et al., 2012; Imai et al., 2012; Linster et al., 2014). This part aims to investigate whether this notion applies to human pandemic viruses from the past.

The mature HA of A/Hong Kong/1/1968, an early isolate obtained during the pandemic in 1968, differs by seven amino acids from the putative avian H3 precursor (Bean et al., 1992; Van Poucke et al., 2015; **figure 4.15**). Two mutations, Q226L and G228S, located in the receptor-binding site, interact directly with sialic acid receptors and are well known to facilitate a switch in receptor-binding specificity from avian (α2,3) to human (α2,6) type receptors (Matrosovich et al., 2000). The remaining five mutations reside in the HA1 subunit. Out of these five substitutions, two (A144G and N193S) reside in the globular head domain at the rim of the RBP. Three substitutions (D62I, D81N and N92K) are located within the vestigial esterase domain which interacts with the HA2 subunit and is involved in the conformational transmission during membrane fusion (Skehel and Wiley, 2000; Xu and Wilson, 2011). Additionally, substitution D81N generates a glycosylation site. Thus, based on their location the five substitutions may influence both receptor-binding and membrane fusion.

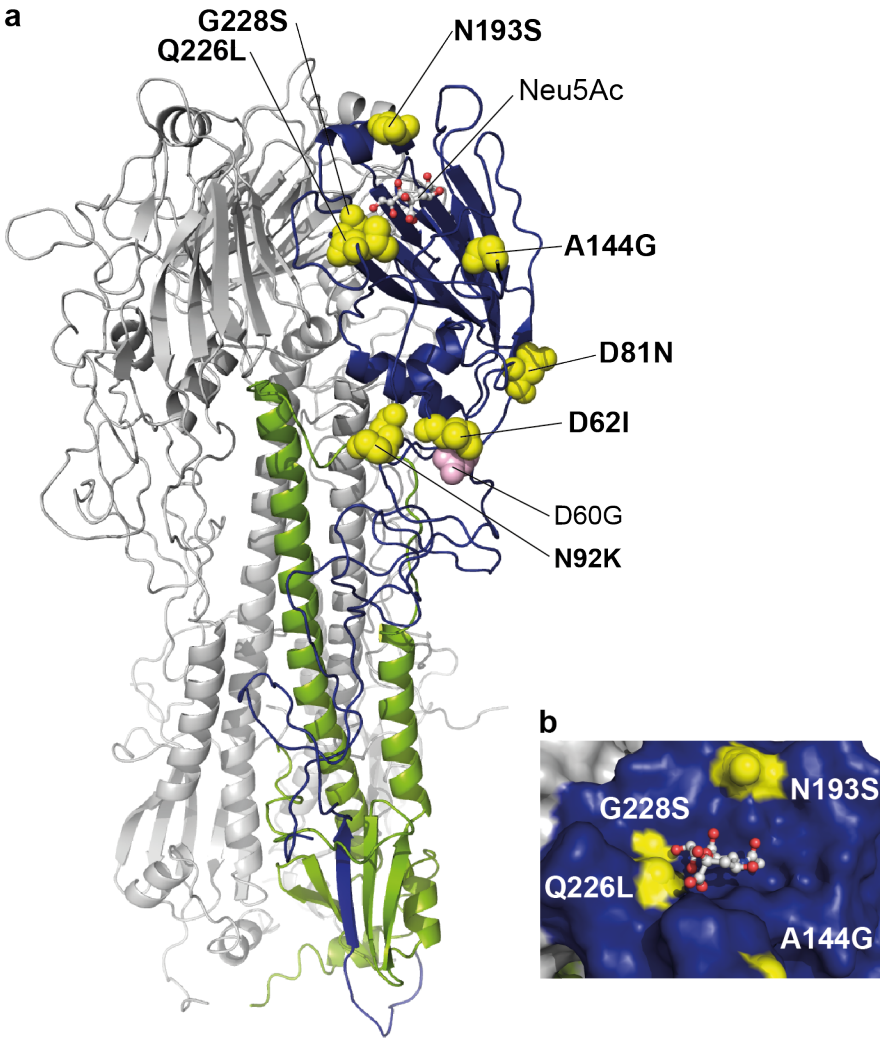

Figure 4.15: HA substitutions separating A/Hong Kong/1/1968 from avian consensus sequence. a *H3 HA monomer (2VIU, protein data bank) is coloured in blue (HA1) and green (HA2). HA mutations acquired during human adaptation are marked in yellow. One mutation (residue 60) acquired during pig-passaging of the avianized mutant rHK/R5 (see the text) is marked in light purple. Amino acid positions are numbered according to H3 numbering.* ***b*** *Inset shows amino acid substitution (yellow) in the receptor-binding pocket. Sialic acid receptor is depicted as ball-stick model.*

To investigate the role of the five substitutions during viral replication, our lab generated a recombinant "avianized" virus (rHK/R5) that contained avian-like amino acids in corresponding 5 positions (Hoffmann et al., 2000). rHK/R5 was then compared with wild type A/Hong Kong/1/1968 (rHK) for their replication and transmission in pigs. Pigs can be efficiently infected by human viruses and show similar pathogenesis to humans (Van Reeth, 2007; Vincent et al., 2014). This makes swine a useful experimental model to study human influenza infection (Meurens et al., 2012; Van Reeth et al., 1998). Our collaborators from Ghent University, Sjouke Van Poucke and Kristien Van Reeth, used the pig model to compared replication efficiency and transmission of rHK and rHK/R5 (**figure 4.16**, Van Poucke et al., 2015). Six- to eight-week-old pigs, serologically negative for influenza, were inoculated

intranasally with 10^6 PFU of either rHK or rHK/R5. After two days six contact pig were introduced. Both, direct contact and airborne transmission was possible under the given housing conditions. Nasal swaps were collected on a daily basis for 9 days postinoculation and postcontact to monitor virus shedding. 50% tissue culture infective doses ($TCID_{50}$) were determined for all swaps by endpoint titration on MDCK cells (described in detail in Van Poucke et al., 2015).

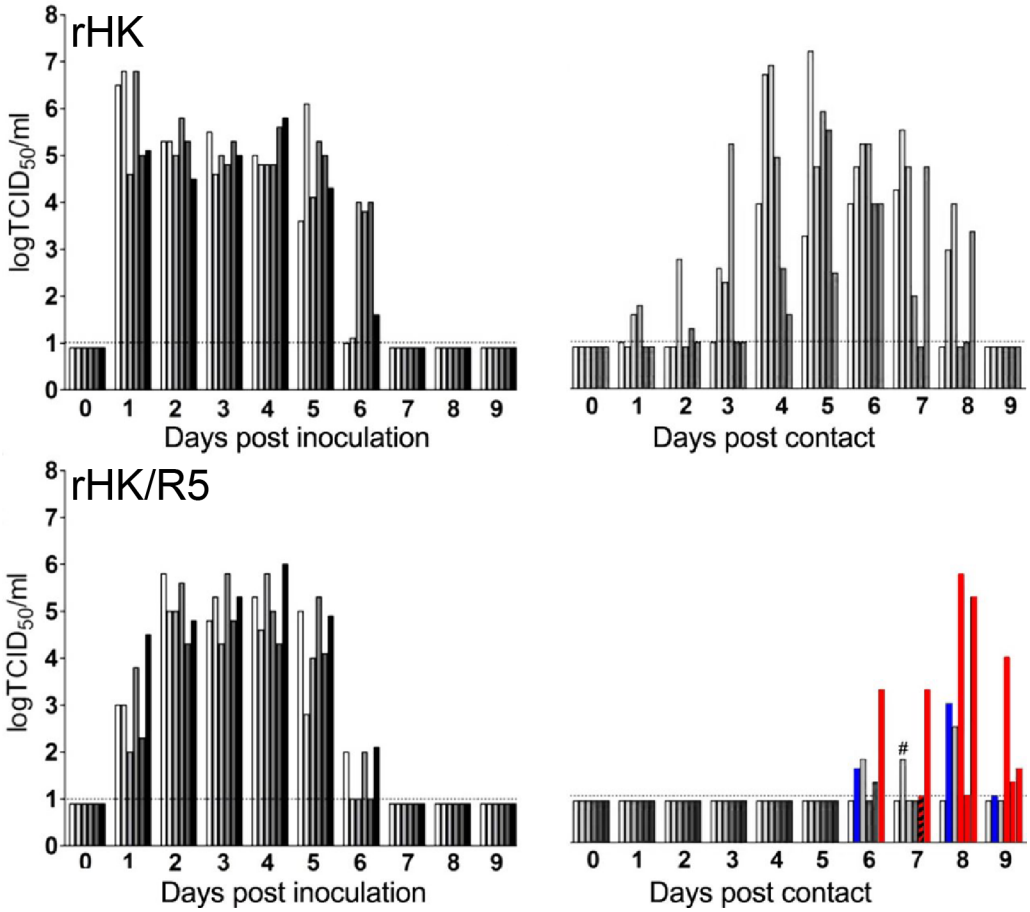

Figure 4.16: rHK and rHK/R5 replication and transmission in pigs. *Six pigs each were infected with either rHK (top panel) or rHK/R5 (bottom panel) and cohoused with six contact animals starting from day two post inoculation. Swap samples were taken from inoculated (left bars) and contact animals (right bars) daily, followed by $TCID_{50}$ titration. The detection limit (10 $TCID_{50}$/ml) is indicated as horizontal dotted line. Contact pigs with mutated HA are indicated in blue (T206I), red (D60G) and red with black stripes (D60G/D). #, virus-positive sample which was not sequenced.*

No difference in virus shedding was observed between pigs inoculated with rHK and rHK/R5 (**figure 4.16**). The wild type rHK virus shows sustained transmission in pigs, whereas no transmission could be observed for rHK/R5. Viruses from virus-positive contact animals in the rHK/R5 group were sequenced. Viruses isolated from four of five contact pigs contained an additional mutation, D60G or T206I. Residue 206 is located in the trimeric interface between single HA monomers and was not further investigated in this study. The D60G mutation

occurred in the vestigial esterase domain in close proximity of the I62R mutations which separates rHK/R5 from rHK (**figure 4.15**). The virus exhibits the D60G (rHK/R5+1) together with the pandemic wild type rHK and the avianized rHK/R5 were characterised regarding receptor-binding and HA-mediated membrane fusion activity.

The receptor-binding to resialylated fetuin containing avian (α2,3) or human (α2,6) type sialic moieties was investigated as described in **3.3.13**. The pandemic rHK wild type virus and the two mutants rHK/R5 and rHK/R5+1 show predominant binding to α2,6 fetuin but still bind fetuin harbouring α2,3 sialic acids (**figure 4.17a**). Thus, introduction of the five avian amino acids in HA of rHK does not alter receptor-binding specificity but slightly increases binding avidity to both α2,3 and α2,6. The additional mutation detected during pig transmission does not further change receptor-binding avidity of rHK/R5+1.

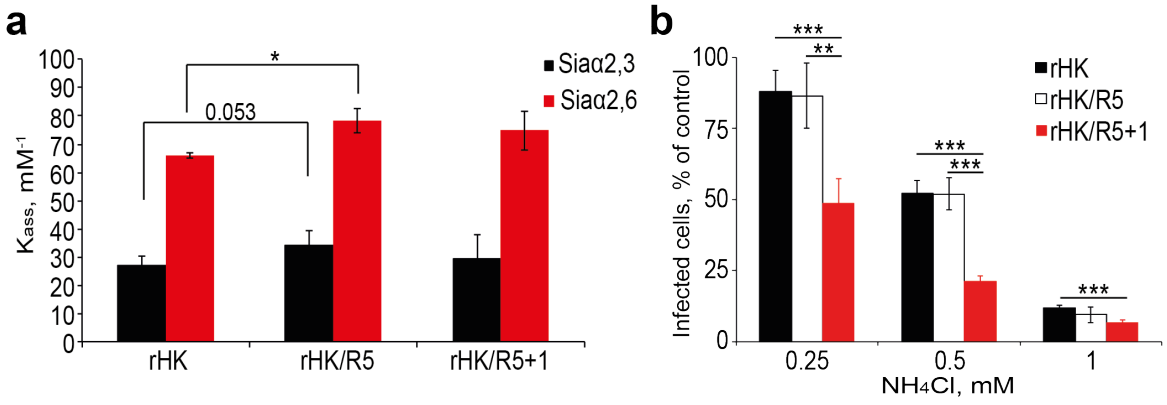

Figure 4.17: Receptor-binding and susceptibility against NH_4Cl of recombinant A/Hong Kong/1/1968 derived viruses. a *Association constants of rHK, avian-like rHK/R5 or pig-passaged rHK/R5+1 for fetuin presenting either α2-3 (black bars; 3fet) or α2-6 (grey bars; 6fet) sialic acids are shown. Error bars represent standard deviation of the data from two independent experiments. *, $P < 0.05$.* ***b*** *Inhibition of virus infection by 0.25 mM NH_4Cl. Viruses were mixed with 0.25, 0.5 and 1.0 mM NH_4Cl followed by MDCK cells infection. Remaining infectivity is displayed as percentage of infection without NH_4Cl. Values resulted from two independent experiments, each performed using three replicates. **, $P < 0.005$; ***, $P < 0.0005$.*

Three of the five mutations (D61I, D81N and N92K) as well as the pig derived D60G mutation are located in the HA vestigial esterase domain between globular head and stalk region. Thus, an effect on HA receptor-binding is unlikely. To investigate the role of these mutations on HA-mediated fusion activity, susceptibility to inhibition by NH_4Cl during viral cell entry was determined (see chapter **3.3.5** and **4.1**). No difference in sensitivity to NH_4Cl was observed between the human wild type rHK and rHK/R5 (**figure 4.17b**). The infectivity of both viruses is inhibited to a comparable degree using three different NH_4Cl concentrations (0.25 mM, 0.5

mM and 1.0 mM). In contrast, the pig-passaged virus HK/R5+1 displayed a significant more sensitive phenotype at all concentrations.

These findings suggest that the five avian substitutions do not alter the dependency on endosomal acidification. The additional mutation 60G in HK/R5+1 results in a stronger reliance on endosomal acidification during viral entry. This suggest that non-optimal membrane fusion activity may have contribute to the inefficient transmission of rHK/R5 in pigs.

4.3.3 HA-mediated membrane fusion activity of zoonotic A/Shanghai/2/2013 (H7N9) isolated from humans

Since May 2013, a H7N9 virus has infected more than 100 people in several provinces in south-eastern China. This virus had been circulating in poultry before crossing barrier species. Typical for zoonotic viruses, more than 75% of the infections were related to poultry contact and only little human-to-human transmission was observed (Gao et al., 2013). This virus shows dual receptor specificity binding both human and avian type receptors (Watanabe et al., 2013). As previous sections of this work demonstrate that membrane fusion properties change during interspecies transmission (section **4.1** and **4.2**), it was tempting to examine fusion activity of the human H7N9 isolate A/Shanghai/2/2013.

HA and NA of the zoonotic H7N9 virus show high sequence homology to two South East Asian avian viruses A/duck/Zhejiang/12/2011 (H7N3) and A/wild bird/Korea/A14/2011 (H7N9), respectively. The internal genes are likely derived from a closely related H9N2 A/brambling/Beijing/16/2012-like virus, following reassortment (Gao et al., 2013). In order to predict potential mutations occurred prior to and during emergence of the zoonotic H7N9 virus, HA sequences of A/Shanghai/2/2013 and A/duck/Zhejang/12/2011 were compared. Seven amino acid differences (**figure 4.18**) separate both HAs. Five of them (D174S, T179V, G186V, I202V and Q226L; H3 numbering) are located in the HA globular head domain. The mutations G186V and Q226L were identified to alter receptor-binding specificity from avian type to human type receptors (Watanabe et al., 2013). Further, two mutations ($T71_2N$ and $N116_2D$) are located in the HA2 stalk domain and may influence HA fusion and stability properties.

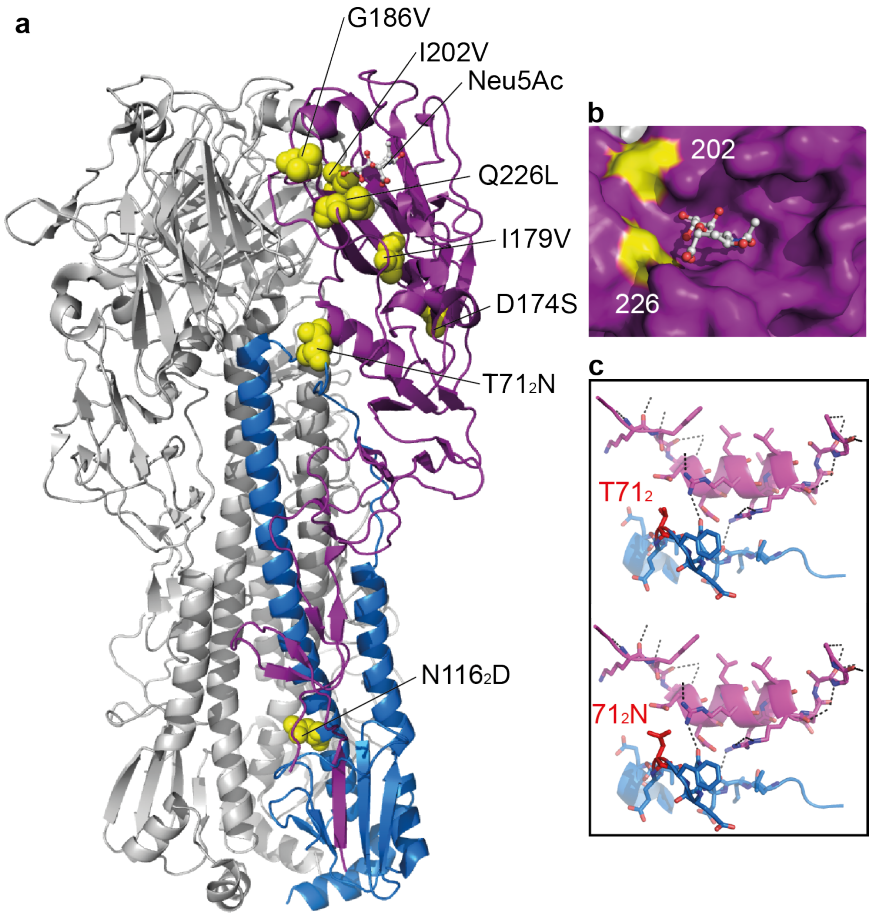

Figure 4.18: Amino acid differences between A/Shanghai/2/2013(H7N9) and a putative precursor in wild birds. a *Mutations in HA1 (purple) and in HA2 (blue) are depicted in yellow. Amino acid positions are numbered according to H3 numbering and shown using H7 X-ray crystallisation data (4LN8; protein data bank).* ***b*** *H7 receptor-binding pocket binding sialic acid. Adjoined mutations are shown in yellow.* ***c*** *$T71_2N$ (red) Interaction at the HA1-HA2 interface.*

To investigate this hypothesis, the A/Shanghai/2/2013 HA was synthesised (GeneScript™) and the substitutions $T71_2N$ and $N116_2D$ were reverted to the avian consensus sequence independently or in combination with each other by site directed mutagenesis. The wild type and mutant A/Shanghai/2/2013 HAs been cloned into pCAGGS expression plasmids and were used to determine pH of syncytia formation as described previously (method section **3.3.11**).

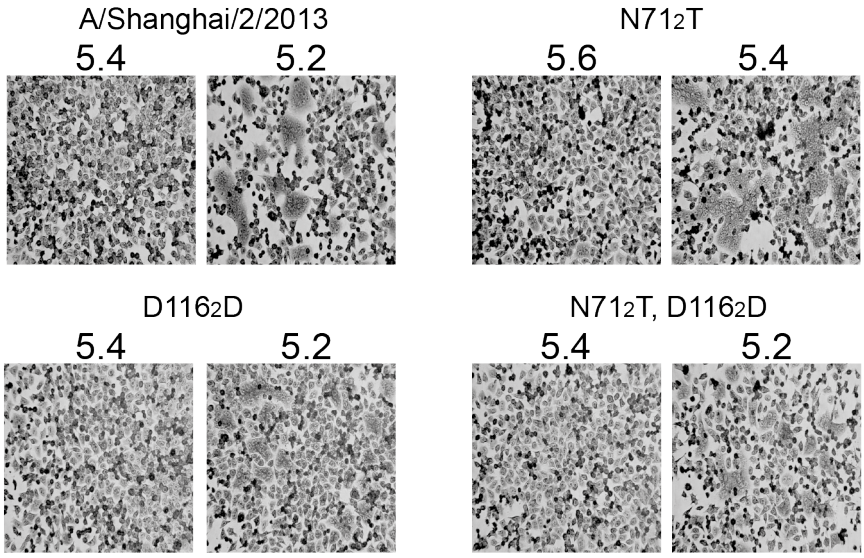

Figure 4.19: Syncytia formation of H7N9 HA-expressing HeLa cells. *HeLa cells were transfected with plasmids coding for A/Shanghai/2/2013 or mutant HA. After transfection, cells were incubated in low pH buffers and cells were stained using Giemsa dye. Micro-photographs were taken at 300x magnification.*

The HA of the human isolate A/Shanghai/2/2013 induced cell-to-cell fusion starting at pH 5.2 (**figure 4.19**). Introduction of the avian type threonine (T) into position 71_2 led to an increase in the pH of fusion induction by 0.2 pH units. No alteration in the pH fusion was observed after introduction of the avian type asparagine (N) at position 116_2. Surprisingly, the combination of both mutations negates the effect of 71_2T, and HA initiates fusion at pH 5.2. This result suggests that 71_2N stabilized the HA of A/Shanghai/2/2013 by decreasing the pH-optimum of fusion and may have contributed to effective replication in humans.

5 Discussion

Over the past 100 years four influenza pandemics have cost millions of lives and have caused a high economic damage worldwide. These latest pandemics arose from viruses circulating in wild aquatic birds. Intermediate hosts, most likely domestic animals such as pigs and different poultry species, were thought to have participated in avian-to-human transmission. Thus, intermediate hosts may play a crucial role in influenza A virus transmission to humans as they act as a link between natural wild life and urban environments (Neumann and Kawaoka, 2015; Shelton et al., 2013).

However, host range restriction is not yet fully defined. A variety of adaptive mutations in the viral polymerase and glycoproteins, HA and NA, are described to be essential to overcome species barriers. Amongst them, mutations in HA that define receptor specificity are studied the best. Domestic pigs and some poultry species express human-like sialic acid receptors on the respiratory epithelia. This makes HA adaptation of avian viruses to human-type receptor-binding more likely, promoting effective transmission to humans (Skehel and Wiley, 2000). Recently, HA-mediated fusion and viral stability were identified to restrict inter- and intraspecies transmission under laboratory conditions (Herfst et al., 2012; Imai et al., 2012). As effective transmission is essential for virus circulation in humans, a certain viral stability evolves during pandemic emergence. Thus, characterising the stability of viruses from different hosts and subtypes may contribute to predict viral pandemic potential.

This work investigates the role of membrane fusion activity and HA-stability as potential host restriction factors during interspecies transmission using several approaches and models. First, fusion activity of H1N1 avian-like swine viruses and closely related avian viruses was studied. In the second part alterations in HA during adaptation of an avian virus to pigs during laboratory experiments were characterised. The third part concentrates on potential differences in the pH optimum of fusion activity between closely related poultry and aquatic bird viruses of the same subtype. Finally, membrane fusion activity and HA stability of pandemic and zoonotic viruses were studied using cell-based fusion assays.

5.1 Avian-like swine H1N1 viruses have a higher pH of membrane fusion activity than their avian precursors

Comparison of pH optima of HA-mediated fusion and HA stability of closely related avian and avian-like swine viruses was performed. The results demonstrated that avian-like swine viruses differ from their avian precursors in terms of a decreased susceptibility to the lysosomotropic agent ammonium chloride (**figure 4.2a**). In agreement with this finding, the HA of swine viruses possesses a higher pH of hemolytic activity when compared to avian viruses (**figure 4.2b**), reflecting a higher pH optimum of HA-mediated membrane fusion activity (**figure 4.2c**). These results confirm similar observation previously made comparing fusion properties of a limited collection of avian and swine viruses (Scholtissek, 1985).

Sequence analysis highlighted eight amino acids that are different between the tested avian and avian-like swine viruses (**figure 4.4**). These residues differ distinctly between HAs from both species and substitutions may emerge during avian-to-swine transmission. Among them, the swine-like amino acid (113_2F) was identified to raise the pH of fusion induction by 0.2 pH units when introduced into an avian HA (**figures 4.6** and **4.7**).

In order to reproduce the evolution of avian viruses during adaptation to pigs, a typical avian virus (A/duck/Bavaria/1/1977) was passaged in pigs by colleagues at the FLI (Riems, Germany) and NVRI (Pulawy, Poland). This work demonstrates that the resulting viruses obtained after passage 15 and 19, respectively, had acquired human-type receptor specificity but maintained binding to avian-type receptors (**figure 4.9**). Furthermore, a decrease in HA stability after treatment at low pH conditions for both pig-passaged viruses could be detected as observed for natural avian and swine isolates (**figure 4.10**). Thus, both pig-passaged viruses display an intermediate receptor-binding phenotype between avian and swine viruses. Nevertheless, the acquired mutations do not lead to increased replication efficiency in HTBE cultures for these viruses (**figure 4.11**). It is possible that the composition of internal genes or imbalance in HA/NA activity restrict replication in human bronchial epithelium. Taken together, the pig-passaged viruses show a switch in the receptor specificity and alteration of HA stability. The observed genetic and phenotypic changes agree nicely with the analysis of natural avian-to-swine transmission (section **4.1**). Nevertheless, this does not seem sufficient to increase replication efficiency in HTBE cultures and does not confer consistent transmission in pigs.

The pH optimum of HA-mediated fusion, as well, influences the timing and intracellular location of viral cell invasion after internalization. During lysosome maturation, endosomes

undergo continued acidification by H^+-ATPases (Jefferies et al., 2008). Thereby, early endosomes possess a mildly acidic environment (pH 6.5 to 6), being further acidified during maturation to late endosomes (pH 5.5 to 5.0) and finally to lysosomes (pH 5.0 to 4.6). Influenza A viruses are believed to fuse at the stage of the late endosome where the endosomal pH drops to pH 5.0 (Grove and Marsh, 2011; Maxfield and Yamashiro, 1987; Sun and Whittaker, 2013). The presented results show that porcine viruses can fuse with the endosomal membrane and may enter the cytoplasm already at earlier stages during endosome maturation. Early endosomes are mostly located in the peripheral regions of the cell (Reviewed in Huotari and Helenius, 2011). Consequently, released RNPs reside longer in the cytoplasm until nuclear import. This may lead to a more efficient recognition by compounds of the innate immune response such as RIG-I. In fact, RIG-I is able to recognize incoming RNPs and binding is sufficient to establish an antiviral state within the cell (Ehrhardt et al., 2010; Weber et al., 2015). On the contrary, the early escape from the maturing endosome may prevent viral recognition by endosome-associated innate immune response proteins, like Toll-like receptors (TLRs), and minimize the effect of antiviral proteins such as IFITMs (Gerlach et al. in preparation). At the time, little is known about the presence and distribution of antiviral proteins in endosomes. In addition, information on how fast endosomal acidification takes place among avian, swine and human cells is limited. Therefore, further studies on differences in cellular physiology and innate immune responses among different host species are needed to clarify the importance of this potential immune evasion mechanism.

There is evidence that cell lines from different species differ in the degree of endosomal acidification. For example, Vero cells were found to possess less acidified endosomes compared to MDCK cells. As a consequence, avian and human influenza viruses display a decreased replication efficiency and evolve a higher pH optimum of fusion after several passages in Vero cells (Murakami et al., 2012). It is possible that porcine endosomes harbour a smaller number of H^+-ATPases resulting in a lower endosomal acidification. This would cause a higher endosomal pH and would make a higher pH of fusion induction necessary, as was observed for viruses after natural and experimental swine adaptation. To further analyse this hypothesis, the expression and activity of H^+-ATPases in different species has to be investigated in more detail.

The pH of HA-mediated fusion is strongly related to viral stability in the environment. In fact, receptor-binding mediated by HA is abolished when fusion is induced. Consequently, the HA conformational change in the absence of a target membrane leads to virus inactivation (Skehel and Wiley, 2000). Indeed, infectivity of swine viruses is inhibited when treated at low pH prior

to cell culture infection compared to avian viruses (**figure 4.2c**). The high pH optimum of fusion therefore results in decreased environmental stability of swine viruses. This can affect both, viral stability in target tissue prior to infection, and efficiency of viral transmission between individuals. As the site of infection varies among hosts, changes in HA stability may be a result of adaptation to the new environmental conditions. Influenza A viruses infect the epithelium of the gastro-intestinal tract in wild birds, whereas they target the respiratory epithelium in mammals. Limited analyses of the pH conditions within the respiratory tract of humans and swine describe the human epithelium as slightly acidic (pH 5.5 – 6.5) whereas the swine respiratory tissue has a more neutral pH (pH 6.93) (Fischer and Widdicombe, 2006; Washington et al., 2000). To date, the pH conditions in the avian gastro-intestinal tract are poorly investigated. Adaptation to tissues that are acidified to a different degree may have contributed to differences in the pH optimum of membrane fusion between avian and swine viruses. One can speculate that the relatively neutral pH within the porcine respiratory tract allows a higher pH optimum for swine viruses to promote infectivity.

Efficient transmission among individuals is a crucial step in the viral life cycle and with this a prerequisite for pandemic emergence. Therefore, viruses have to acquire a certain stability to facilitate transmission in a given medium (e.g. water, air or body fluids). Avian viruses require a sufficient stability for transmission as they spread via the fecal-oral route including short-time persistence in water, which is slightly acidic and may lead to an reduced viral stability (Brown et al., 2009; Reed et al., 2010). The introduction into mammals results in a different viral transmission route as a consequence of different sites of infection. Mammalian viruses, in contrast to wild bird viruses, replicate in the respiratory tract. Consequently, either direct contact or airborne transmission via respiratory droplets is preferred for mammalian influenza A viruses (Sorrell et al., 2011). As a prerequisite for airborne transmission in humans and ferrets a switch of avian to human type receptor specificity is needed, as human type α2,6-linked sialic acids are predominant in the upper human respiratory tract (reviewed in de Graaf and Fouchier, 2014). Nevertheless, two groups demonstrated for H5N1 viruses that a switch in receptor-binding preference alone is not sufficient to facilitate transmission via respiratory droplets in ferrets. Both identified additional mutations that increase receptor-binding avidity and create a new glycosylation site in the HA head domain. In addition the ferret-transmissible viruses acquired mutations stabilizing HA, indicating a role of HA stability in transmission (Imai et al., 2012; Linster et al., 2014). Compared to viruses of other subtypes, H5 virus belong

to the least stable influenza viruses (Galloway et al., 2013). As HA stability varies between different virus isolates and subtypes it remains unclear whether adaptation of HA stability and fusion activity is a general feature of interspecies transmission (DuBois et al., 2011).
In contrast, low stability H5N1 viruses are able to transmit between ferrets via direct contact without mutations increasing their stability (Lowen et al., 2006; Maines et al., 2006; Schrauwen and Fouchier, 2014) suggesting less strict stability requirements for direct transmission. H5N1 viruses studied by others (DuBois et al., 2011; Galloway et al., 2013) and swine viruses tested in this work show a comparable low stability. Even though limited studies on transmission of these viruses in pigs were performed, the low HA stability of swine viruses makes airborne transmission unlikely. It is possible that a high population density in pig farming houses with close contact among animals contributes to direct contact transmission. Consequently, HA does not necessarily need to possess a high stability to allow transmission. The fast reduction of HA stability during emergence of the EAsw lineage suggests an evolutionary advantage during avian-to-swine transmission. It is possible that a decreased stability confers a higher replication efficiency in the porcine lung.
In summary, swine viruses differ from their avian precursors by a higher pH optimum of fusion and a lower HA stability. Additionally, during laboratory avian-to-swine transmission a decrease in HA stability, in addition to the switch in receptor specificity, was identified to rapidly occur in the course of pig adaptation. The combination of different pH requirements for replication, immune evasion, and transmissibility in different host species may represent a host range barrier.

5.2 HA membrane fusion activity of H7 viruses differs between host species

To investigate differences in the HA-mediated fusion and stability properties between different avian species, wild bird and poultry H7 viruses were compared. Among poultry viruses, H7 viruses represent the most abundant subtype within poultry populations (Alexander, 2007; Lam et al., 2013). The presented results show that Eurasian poultry H7 viruses are less dependent on endosomal acidification during cell entry (**figure 4.13a**), possess a higher pH optimum of hemolytic activity (**figure 4.13b**) and are less stable than viruses from wild birds (**figure 4.13c**). Eurasian H7 viruses displayed the highest pH of fusion and the lowest HA stability among viruses from all subtypes including North American H7 viruses. This difference does

not depend on the viral origin from wild birds or poultry species. One hypothesis is that this is the result of a continued exchange of viruses between wild birds and poultry species in Eurasia (Lebarbenchon and Stallknecht, 2011).

North American H7 viruses possess a higher stability (**figure 4.13**) and display reduced virulence in mammals when compared to Eurasian H7 viruses (Belser et al., 2009). In mice, influenza viruses displaying a high pH of fusion show a higher virulence and pathogenicity than stable viruses with lower pH of fusion. H7 viruses of the Eurasian lineage are able to replicate efficiently in the respiratory tract without preceding adaptation (Belser et al., 2007; de Wit et al., 2005). This finding and the wide circulation of H7 viruses in domestic animals raise public health concerns. Nevertheless there is no evidence that humans are more susceptible to infection with H7 viruses than to viruses of other subtypes (Dybing et al., 2000), even though some North American H7 viruses are more adapted to humans based on their receptor-binding profile (Belser et al., 2009).

Despite the fact that viruses with H5 HA were not investigated in this study, other studies indicate that H5 viruses show a similar stability to the tested H7 viruses (Russell, 2014). Both H5 and H7 viruses have caused human infections in the past. However, human infection with neither of them was accompanied by airborne human-to-human transmission, as most of the human infections can be traced back to direct contact to poultry (Gao et al., 2013). It is possible that airborne transmission requires a certain viral stability in order to be effective. Accordingly, the low stability of H7 and H5 viruses may obstruct airborne transmission and potentially favours direct contact transmission.

The availability of early human H7N9 isolates from the recent outbreak in 2013 in Southeast Asia provides an opportunity to study potential changes in H7 virus stability during zoonotic infection. Early characterisation of these isolates revealed a dual receptor specificity (Watanabe et al., 2013). However, these viruses bind human-type receptors with low affinity. It is not clear whether the virus acquired human type receptor-binding before or shortly after human infection (Zhou et al., 2013). The human H7N9 can replicate efficiently in human trachea and lung cultures (Zhou et al., 2013). In the presented work, two mutations ($T71_2N$ and $N116_2D$) in the HA stalk were identified to separate the human isolate from the closest known avian virus. The avian-like amino acids were introduced into the HA of the human isolate and HA fusion activity was characterised. The exchange of threonine by asparagine in residue 71_2 results in an increase in the pH of fusion induction **(figure 4.19)**. This indicates that A/Shanghai/2/2013 acquired a more stable HA than the closest avian virus prior to or following human infection. However, the zoonotic H7N9 is still less stable than known human-transmissible viruses. This fact could

be responsible at least in part for the inefficient human-to-human transmission during the recent H7N9 outbreak (Gao et al., 2013). The limited airborne transmission suggests that this virus is not yet fully adapted to humans and virus stability may increase with continued evolution in humans. A similar observation was made for currently circulating descendants of the swine-origin 2009 pandemic virus. Directly after transmission to humans, the 2009 pandemic virus HA exhibits a lower stability than virus isolates of earlier pandemics (**figure 4.14**). Even though the virus readily transmitted among humans, continued circulation in the population resulted in increased stability (Cotter et al., 2014).
Since a higher viral stability may be accompanied by a higher transmission efficiency, further studies involving recent human H7N9 are needed to elucidate the H7N9 virus evolution.

The results presented in this thesis show that Eurasian H7 viruses from domestic birds possess a low environmental stability. As all known pandemic viruses show a high stability, which is believed to contribute to airborne transmission, the low stability of EA H7 viruses may hamper establishment in humans. Furthermore, the fusion modulating mutation in the human H7N9 in in addition to human type receptor-binding may have increased viral fitness in humans compared to typical poultry viruses. Nevertheless, the H7N9 virus is still not as stable as human adapted viruses and, in combination with other factors, this seems to limit transmissibility. The H7N9 outbreak in 2013, the circulation of several H7 viruses ready to infect humans, and the wide genetic compatibility of H7 HA with several NA subtypes illustrate the potential of H7 viruses to cause a future pandemic.

5.3 Membrane fusion activity and receptor-binding avidity of the HA of A/Hong Kong/1/1968

The HA of the early pandemic isolate A/Hong Kong/1/1968 differs by seven amino acid substitutions from its predicted avian H3 precursor. Two of these mutations (Q222L and G226S) are known to facilitate the switch in receptor-binding specificity from avian to human type binding (Rogers and Paulson, 1983; Viswanathan et al., 2010). Results of the present work indicate that the five remaining mutations (R61I, D81N, N92K, A144G and N193S) when reverted to the avian sequence (rHK/R5), increase receptor-binding avidity to both human and avian type sialic acid receptors (**figure 4.17a**). No effect on dependency on acidification during cell entry could be observed (**figure 4.17b**). This is in contrast to poultry H5 viruses that need

to change HA-stability for adaptation to humans (Herfst et al., 2012; Imai et al., 2012). Whereas most avian H5 viruses possess a low stability, the avian precursor of the 1968 pandemic virus was sufficiently stable to replicate in, and transmit between humans. Thus, avian viruses may or may not require changes in fusion pH and stability depending on their origin and properties. Both, rHK and rHK/R5 are able to replicate in pigs (**figure 4.16**), but unlike the human wild type, rHK/R5 is not able to transmit efficiently between pigs (Van Poucke et al., 2015). As discussed in section **1.7**, changes in the receptor specificity, binding avidity, and stability were required for airborne transmission of two different H5N1 avian viruses in ferrets (Imai et al., 2012; Linster et al., 2014). Alteration of receptor-binding avidity but no difference in HA stability have occurred during the emergence of the 1968 pandemic virus. In this work, the HA glycosylation status was not studied, but sequence analysis identified the mutation D81N to remove a potential glycosylation site within the HA. It is likely that the removal of this glycosylation site contributes to changes in binding avidity. Further studies using single point mutants are needed to understand the role of this position during the emergence of the 1968 pandemic virus.

After several rounds of replication in pigs an additional mutation in the HA occurred, D60G. This position is structurally in close proximity to the reverted amino acid 62 and may complement its function. A phenotypic characterisation of the virus harbouring D60G revealed a lower pH of fusion during cell entry but displayed no altered receptor-binding avidity when compared to rHK/R5. It is likely that the lower pH of membrane fusion results in a higher HA stability. This finding disagrees with the increase of fusion pH observed during natural avian-to-swine transmission shown in section **4.1**. It is possible that this discrepancy is a result of different environmental settings. Differences in housing conditions (e.g. space per pig) in piggeries and laboratories may favour either direct or airborne transmission, which may influence stability necessities for transmission. However, the specific reason for this disagreement is not clear and further studies are needed for elucidation. Nevertheless, selection of the D60G mutation during transmission suggests that it compensates at least partially for avian-like amino acids in rHK/R5.

As human viruses transmit via respiratory droplets (Belser et al., 2013; Tellier, 2006) and infect cells in the acidic environment of the human airway cavity (England et al., 1999; Washington et al., 2000), they may require a more stable HA. Our results indicate that five non-226/228 mutations in the HA served to optimize viral receptor-binding avidity but have no influence on fusion and stability properties. Taking into account that the five mutations arose during adaptation to humans, HA stabilization seems to be important for the emergence of this

pandemic virus and may facilitate human-to-human transmission. To investigate the role of individual substitutions, single point mutants should be studied in the future.

In summary, this work identifies swine viruses and Eurasian poultry H7 viruses to have a higher pH optimum of HA-mediated fusion and a lower HA stability than both wild bird and human viruses. These differences seem to influence interspecies transmission but most likely do not prevent general infection by contact transmission. Considering these findings, HA stability seems to be less important for individual infections, but required for viral spread within a population. Analysis of mutations acquired during transmission of the pandemic A/Hong Kong/1/1968 virus and the human H7N9 virus shows similar adaptation processes, indicating modification of HA fusion and stability properties. Therefore, viral replication and transmission may depend on optimal stability and fusion properties which represent potential host range restriction factors. However, many other factors, which have yet to be determined, characterise these optimal fusion properties. The findings presented in this work prompt further studies on membrane fusion characteristics in different host species and their potential effect on the zoonotic and pandemic potential of influenza A viruses.

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7 List of figures and tables

8 Appendices

8.1 Abbreviations

A	Absorption
ATP	Adenosine triphosphate
BHK	Baby hamster kidney
BSA	Bovine serum albumin
c	Concentration
CI	Confidence interval
cm2	Square centimetre
Crm1	Chromosomal maintenance 1
cRNA	complementary RNA
CT	Cytoplasmic tail
CMV	Cytomegalovirus
DMEM	Dulbecco´s modified Eagle´s medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EA	Eurasian
EAsw	Eurasian avian-like swine
EDTA	Ethylenediaminetetraacetic acid
e.g.	exempli gratia (for example)
ELISA	Enzyme-linked immunosorbent assay
ESWI	European Scientific Working group on Influenza
et al.	et alii (and others)
FAO	Food and Agriculture Organization
FCS	Fetal calf serum
FFU	Focus forming units
FITC	Fluorescein isothiocanate
FLI	Friedrich Loeffler Institute
Gal	Galactose
GP	Glycoprotein
h	Hour
HA	Hemagglutinin
HAD	Hemadsorption site

HAT	Human airway trypsine-like protease
HAU	Hemagglutination units
HEF	Hemagglutinin-esterase-fusion protein
HEK	human embryonic kidney
HeLa	Henrietta Lacks
HPAI	High pathogenic avian influenza
hRBC	human red blood cells
HRP	Horse radish peroxidase
HTBE	Human tracheobronchial epithelial cells
IC	Inhibition concentration
IFITM	Interferon induced transmembrane protein
IFN	Interferon
IgG	Immunoglobulin G
Kb	Kilo-base pair
LB	Lysogeny broth
LPAIV	Low pathogenic avian influenza virus
M	Mole
M1/2	Matrixprotein 1/2
mA	Milliampere
MCM	Minichromosome maintenance compex
MDCK	Madin-Darby canine kidney
MEM	Minimum essential medium
min	Minute
ml	Millilitre
mM	Millimole
MOI	Multiplicity of infection
mRNA	messenger RNA
NA	Neuraminidase
NAm	North American
NCBI	National Centre for Biotechnology Information
NEP	Nuclear export protein
NES	Nuclear export sequence
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
ng	Nanogramme
NLS	Nuclear localization sequence
nm	Nanometre
NP	Nucleoprotein

NS1/2	Non structural protein 1/2
NVRI	National veterinary research institute
NXF1	Nuclear RNA export factor 1
OIE	World Organisation for Animal Health
PA	Polymerase acidic
PB1	Polymerase basic 1
PB2	Polymerase basic 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
Pfu	Pyrococcus furiosus
pH	Potentia hydrogenii
RBP	Receptor-binding pocket
rER	Rough endoplasmic reticulum
RIG-I	Retinoic acid-inducible gene 1
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SAP	Shrimp alkaline phosphatase
sec	Second
SGP	Sialylglycopolymers
SP	Signal peptide
TBE	Tris-Borate-EDTA
$TCID_{50}$	50% Tissue culture infective dose
TM	Transmembrane domain
TMB	3,3′,5,5′-Tetramethylbenzidine
TMPRSS2/4	Transmembrane protease serine S2/4
TPCK	Tosyl phenylalanyl chloromethyl ketone
TREX	Transcription export complex
TSS	Transformation and storage solution
µg	Microgramme
µl	Microlitre
USA	United States of America
V	Volts
vRNA	viral RNA

WHO	World Health Organisation
wt	wild type

8.2 Amino acid abbreviations

Amino acid	3-letter code	1-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Try	W
Tyrosine	Tyr	Y
Valine	Val	V

8.3 Publications

Baumann J, Kouassi NM, Foni E, Klenk HD, Matrosovich M. 2015. H1N1 Swine Influenza Viruses Differ from Avian Precursors by a higher pH Optimum of Membrane fusion. **J Virol.** 90(3):1569-77. doi:10.1128/JVI.02332-15.

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Linster M, van Boheemen S, de Graaf M, Schrauwen EJA, Lexmond P, Mänz B, Bestebroer M, **Baumann J**, van Riel D, Rimmelzwaan GF, Osterhaus ADME, Matrosovich M, Fouchier RAM, Herfst S. 2014 "Identification, Characterization, and Natural Selection of Mutations Driving Airborne Transmission of A/H5N1 Virus." **Cell**; 157(2):329-39. doi: 10.1016/j.cell.2014.02.040.

Arias A, Watson SJ, Asogun D, Tobin EA, Thorne L, **Baumann J** et al. 2015. Rapid outbreak sequencing of Ebola virus in Sierra Leone identifies transmission chains linked to sporadic cases. **Lancet**. Submitted.

Caroll MW, Matthews DA, Hiscox JA, Elmore MJ, Pollakis G, Rambaut A, **Baumann J** et al. 2015. Temporal and spatial analysis of the 2014-2015 Ebola virus outbreak in West Africa. **Nature** 524(7563):97-101. doi: 10.1038/nature14594.

8.4 Presentations

Baumann J, Kouassi NM, Foni E, Klenk HD, Matrosovich M.
"Host-specific differences in membrane fusion activity of H1N1 influenza A viruses." VIII Options for the Control of Influenza. September 5th – 10th 2013 in Cape Town, South Africa.
Oral presentation

Baumann J, Kouassi NM, Klenk HD, Matrosovich M.
"Differences in the pH optimum of HA-mediated fusion between avian, swine and human influenza viruses" 5th ESWI Influenza Conference. September 14th – 17th 2014 in Riga, Latvia.
Oral presentation

Baumann J, Klenk HD, Matrosovich M.
"Differences in membrane fusion activity of influenza A viruses." 23rd Annual Meeting of the Society for Virology. March 6th – 9th 2013 in Kiel, Germany.
Poster presentation

Baumann J, Klenk HD, Matrosovich M.
"Host-specific differences in membrane fusion activity of influenza A viruses." 22nd Annual Meeting of the Society for Virology. March 14th – 17th 2013 in Essen, Germany.
Poster presentation

Baumann J, Kouassi NM, Klenk HD, Matrosovich M.
"Host-specific differences in membrane fusion activity of influenza A viruses in aquatic birds and pigs." 3rd International Influenza Meeting. September 2nd – 4th 2012 in Münster, Germany.
Poster presentation

8.5 Curriculum Vitae

Personal Information

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Work Experience

Since Jul 2015	Scientific Staff Institute of Virology; Philipps University Marburg
Jun - Jul 2015	Team Leader WHO Consultant for Ebola Diagnostic European Union Mobile Laboratory Freetown, Sierra Leone
Jan - Feb 2015	WHO Consultant for Ebola Diagnostic European Union Mobile Laboratory Freetown, Sierra Leone
2011 - 2015	Graduate Researcher / PhD Thesis Institute of Virology; Philipps University Marburg
2008	Student Assistant Institute of Molecular Genetics; Martin Luther University Halle-Wittenberg
2006	Student Assistant Institute of Molecular Genetics; Martin Luther University Halle-Wittenberg

Education

2003 - 2010	Diploma in Biosciences Martin Luther University Halle-Wittenberg Diploma thesis: "*Genetische Optimierung der LAC-Expressionskassette zur Generierung immunogener Kluyveromyces lactis Stämme*" ("Genetic optimization of the LAC-Expression system to generate immunogenic *Kluyveromyces lactis* strains")

2002 - 2003	Civilian Service
	St. Elisabeth und St. Barbara Hospital; Halle-Wittenberg
1994 - 2002	General Qualification for University Entrance
	Wilhelm-Ostwald-Gymnasium, Leipzig
1990 - 1994	Primary School
	August-Bebel-Grundschule, Leipzig

8.6 List of academic teachers

My teachers at the Martin-Luther University Halle Wittenberg were:

Anders, Andreesen, Behrens, Bonas, Breunig, Bruelheide, Dorn, Ferenz, Fritsche, Gattermann, Hensen, Jäger, Johanningmeier, Klösgen, Moritz R, Moritz GB, Nies, Reuter, Röser, Sawers, Seliger, Stubbs, Tschuch, Ulbrich-Hoffmann, Wasternack, Weinandy, Weinert.

My teachers at the Phillips University Marburg were:

Klenk, Matrosovich.

8.7 Acknowledgments

First of all, I would like to thank Dr. Mikhail Matrosovich, for the opportunity to do my PhD thesis under his supervision. I cannot appreciate enough the doors he opened for me and everything I learned from him during the last years. His support and the intensive discussions, I believe, made me a better scientist. I am very grateful to have been part of his group.

I gratefully acknowledge the cooperation with the members of the FluPig consortium. In particular, I thank Dr. Sjouke van Poucke and Prof. Dr. Kristien Van Reeth from the University Gent, Dr. Miranda de Graaf, Martin Linster and Prof. Dr. Ron Fouchier from the Erasmus Medical Centre Rotterdam for the opportunity to contribute to several publications. Certainly, I would like to thank Kinga Urbaniak, Andrzej Kowalczyk and Prof Dr. Iwona Markowska-Daniel from NVRI, Pulawy as well as Dr. Jürgen Stech and Prof. Dr. Thomas Mettenleiter for providing pig-adapted viruses for our study.

I would like to thank Prof. Dr. Hans-Dieter Klenk for the weekly discussion and his support allowing me to experience the Hong Kong Pasteur Institute 'Virology course'. For the support during the European Mobile Laboratory missions I thank Prof. Dr. Stephan Becker.

A special thanks goes to Nancy Mounogou Kouassi. It was a pleasure to supervise her during her Bachelor and Master thesis and her enthusiastic work tremendously pushed our project forward.

I would like to thank all other people in the working group of Dr. Matrosovich. Without the patient help and the detailed explanations from Tatyana Matrosovich my start as a virologist would have been way more difficult. Thomas, what should I say? Your 'Rheinische Frohnatur' cheered me up countless times. Thank you for your help, the discussions and your emotional backslappings during these almost five years in the same room. This room would have been way to quite without the rest of the group, Johanna West, Isabel Wendel, Luca Hensen, Volker Czudai-Matwich. Thank you for all the funny hours we spend together.

A big thanks to all colleagues from the other groups and staff at the Institute of Virology, Marburg for the excellent working conditions and all the help and support I experienced from you.

As always without the help of good people achieving goals would be way more difficult. I thank Prof. Dr. Eva Böttcher-Friebertshäuser for the help, the advices and numerous conversations during our lunch and coffee breaks. I want to thank Dr. Lucie Sauerhering for always having an open ear for me. A special 'Thank you!' goes to Ulrike Felgenhauer, who suffered the most correcting my thesis, and to all the other patient and helpful correctors: Dr. Hanna Sediri, Dr. Michaela Gerlach, Thomas Gerlach.

Finally, I would like to thank my family and friends. It is hard to say how much your support mean to me. Thank you for being at my side in good and bad times. The help, love and trust of my parents and my sister always helped me to keep going, from the first day on.

Jule, thank you for the past almost 11 years. Without you I would have got lost long before finishing this thesis.

8.8 Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel

- Host-specific differences in the membrane fusion activity of influenza A viruses. -

im Institut für Virologie unter der Leitung von Dr. Mikhail Matrosovich ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher weder an einem in- oder ausländischen medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt

Teile der vorliegenden Arbeit wurden in folgenden Publikationsorganen veröffentlicht:

Journal of Virology (2x)

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Marburg, den 2016